



DOCTORAL THESIS

**GENETICS, EPIGENETICS, AND GENOTYPE
ENVIRONMENT INTERACTION IN RAS-REARED
ATLANTIC SALMON, *SALMO SALAR***

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Declaration of Authorship

This thesis is the results of my own work, completed wholly or mainly while a candidate for a research degree at this university. Any work which has previously been submitted for any qualification at this or any other institution has been clearly indicated. Where this work has been based off that performed jointly with assistance it been highlighted, with my and other's contributions clearly stated. Attributions to the main sources of help, use of published work (including figures), and quotations of published work have always been clearly stated. Any use of artificial intelligence technologies, stating the intent of such use, has been described. All other work is mine alone.

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Abstract

While use of freshwater net-pens is widespread in Scotland, employment of recirculating aquaculture systems (RAS) for Atlantic salmon aquaculture is increasing. These husbandry environments differ which, due to environmental influences on salmon's life cycle, may impact their commercial production. This study aimed to understand the environmental, genetic and epigenetic factors influencing salmon performance under varied culture systems by: (1) estimating phenotypic and genetic parameters, as well as genotype-environment interactions (GxE) of a population reared in RAS or ambient loch system, (2) studying if manipulation of environmental parameters impacted DNA methylation and gene expression, and (3) assessing whether the freshwater environment impacted salmon performance in saltwater.

Results indicated that RAS-reared smolts were smaller, had lower trait variance, and greater heritability estimates compared to loch counterparts. Moderate GxE were identified for weight, as well as differing genomic associations with phenotypic expression depending on rearing environment. However, environmental manipulation was not observed to impact DNA methylation in a meaningful way, despite clear gene expression changes. In saltwater, despite their smaller size on transfer, RAS-reared post-smolts appeared to grow at a faster rate than loch-reared counterparts while maintaining greater trait heterogeneity. This difference was reflected in low-to-moderate estimates of GxE for growth rate. Temporal patterns in trait heritability also differed with freshwater-rearing environment.

This suggested that although differences in phenotypic performance were observed at the end of freshwater-rearing, these did not negatively impact the RAS population after saltwater transfer. Such conclusions could benefit from further investigation into the genetic regulation of environment-specific traits, economic viability of potential management decisions, and further GxE studies, for example. Furthermore, although differences were observed in phenotypic performance at both freshwater and saltwater phases, this did not appear to result from methylation changes despite gene expression differences, suggesting a possible role of alternative epigenetic mechanisms.

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Publications and Conferences

Publication: Tollervey MJ, Bekaert M, González AB, Agha S, Houston RD, Doeschl-Wilson A, Norris A, Migaud H, Gutierrez AP. Assessing genotype-environment interactions in Atlantic salmon reared in freshwater loch and recirculating systems. *Evol Appl.* 2024 Aug 9;17(8):e13751. doi: 10.1111/eva.13751. PMID: 39131541; PMCID: PMC11310769.

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Abbreviations and Acronyms

5caC: 5-carboxycytosine

5fC: 5-formylcytosine

5hmC: 5-hydroxymethylcytosine

5mc: 5-methylcytosine

AAAD: Aromatic amino acid decarboxylase

AANAT: Arylalkylamin N acetyltransferase

ADG: Average daily gain

ADG_{LENGTH}: Average daily gain for length

ADG_{WBW}: Average daily again for whole body-weight

AICDA: Activation-induced cytosine deaminase

ANOVA: Analysis of variance

APOBEC: Apolipoprotein B mRNA editing enzyme catalytic polypeptide

ATP: Adenosine triphosphate

A_v: additive genetic variance of residual variance

BAM: Binary alignment map

BCP: 1-bromo-3-chloropropane

BER: Base extension repair

Bio_{final}: End biomass per tank (kg)

Bio_{mort}: Mortality biomass (kg)

Bio_{start}: Start biomass per tank (kg)

bp: Base pairs

°C: Degrees Celsius

CF: Continuous feeding

CGV: Coefficient of genetic variance, $CGV = (\sqrt{V_g}/\bar{X}) * 100$

CMS: Cardiomyopathy syndrome

CO₂: Carbon dioxide

CpG: 5'-cytosine-phosphate-guanine-3'

CpGi: CpG islands

CV: Coefficient of phenotypic variance, $CV = (SD_p/\bar{X}) * 100$

CGV_v: Coefficient of genetic variance

CGV_{sd}: Coefficient of standard deviation

DEG: Differently expressed genes

DEG_{800dd}: Differently expressed genes of fish exposed to environmental manipulation at 800dd relative to control conditions and independent of changes observed though time

DEG_{time}: Differently expressed genes through time, identified by a comparison between baseline and control conditions at 800dd

DF: Day feeding

DMC: Differently methylated cytosine

DMC_{800dd}: Differently methylated cytosine of fish exposed to environmental manipulation at 800dd relative to control conditions and independent of changes observed though time

DMC_{gene}: Genes overlapped by differently methylated cytosine

DMC_{time}: Differently methylated cytosine through time, identified by a comparison between baseline and control conditions at 800dd

DMR: Differently methylated region

DNA: Deoxyribose nucleic acid

DNA_{me}: DNA methylation

DNMT: DNA methyltransferases

DW_{feed}: Dry weight (g) of feed provided

DW_{waste}: Dry weight (g) of uneaten feed

EBV: Estimated breeding value

FA: Factor analytic structures

FASTA: Fast-all	K_{sw}: Condition factor measured at the saltwater site
FC_{kg}: Total feed consumed	LCF: Latent common factors
FCR: Feed conversion efficiency	Length_{FW}: Length measured at the freshwater site
FDR: False discovery rate	Length_{sw}: Length measured at the saltwater site
FI_{%BW/day}: Feed intake as a percentage of weight per day	LL: Continuous light photoperiod
FI: Feed intake	LOCO: Leave once chromosome out
FI_{average}: Average feed intake per fish per day	lncRNA: long non-coding RNAs
FPKM: Fragments per kilobase million	LND: Photoperiod with both light and dark periods
FW: Freshwater	LT: Low temperature (~11°C)
FW-SW offspring: Combined samples from FW and SW)	MAS: Marker assisted selection
GCTA: Genome-wide complex trait analysis	MBD: Methyl-binding-domain
GH: Growth hormone	meQTL: Methyl quantitative trait loci
GIFT: Genetically improved farmed tilapia	miRNA: MicroRNAs
GO: Gene ontology	MLM: Mixed linear model
GRM: Genomic relationship matrix	MLM-LOCO: Mixed linear model-leave one chromosome out
GWA: Genome wide association	mRNA: Messenger RNA
GWAS: Genome wide association study	MVN: Multivariate normal
GxE: Genotype environment interaction	NaCl: Sodium chloride
h²: Narrow sense heritability	NaHCO₃: Sodium bicarbonate
HIOMT: hydroxyindole-O-methyltransferase	NCBI: National centre for biotechnology information
HT: High temperature (~15.5°C)	ncRNA: Non-coding RNA
h_v²: Heritability of variance, defined as the accuracy of predicted estimated breeding values of the residual variance	NH₃: Ammonia
IGF-1: Insulin growth factor 1	NH₃⁻: Unionized ammonia
K: Condition factor	NO₂⁻: Nitrite
KEGG: Kyoto encyclopaedia of genes and genomes	NO₃⁻: Nitrate
K_{fw}: Condition factor measured at the freshwater site	NUT: Turbidimetric turbidity unit
	O₂: Oxygen
	OH: Opposite homozygosity
	PCA: Principal component analysis

PiRNA: Piwi-interacting RNAs	SNP: Single nucleotide polymorphisms
PIT Tags: Passive integrated transponder tags	SS: Suspended solids
PNES: Photoneuroendocrine system	SW: Saltwater
POA: Preoptic area	T3: Triiodothyronine
ppt: Part per million	T4: Thyroxine
PST: Parr-smolt transformation	TAN: Total available ammonia
QC: Quality control	TDG: Thymine DNA glycosylase
QGM: Quantitative genetic models	tDMR: Tissue-specific differently methylated region
QTL: Quantitative trait loci	TET: Ten-eleven translocation
RAS: Recirculating aquaculture systems	TIMAP: TGF- β inhibited membrane associated protein
REML: Restricted maximum likelihood	TPOH: Tryptophan hydroxylase
r_g: Genetic correlation	TF: Transcription factor
r_{gxe}: Genotype environment interaction genetic correlation	TGC: Thermal growth coefficient
RISC: RNA-induced silencing complex	TRI reagent: Trizol
RNA: Ribose nucleic acid	tSNE: t-distribution stochastic neighbour embedding
RNase: Ribonuclease	TSS: Transcription start site
r_r: Residual correlation	TTS: Transcription termination site
r_{vm}: Genetic correlation between trait mean and its variance	V_d: Dominance variance
RRBS: Reduced representation bi-sulphite sequencing	V_e: Environmental variance
s.d: Standard deviation	V_g: Additive genetic variance
s.e: Standard error	V_p: Phenotypic variance
SAM (file): Sequence alignment/map	UNCS: University of California Santa Cruz
SAM: Adenosylmethionine	WBW: Whole-body weight
SCN: Suprachiasmatic nucleus	WBW_{FW}: Whole-body weight measured at the freshwater site
SDS: Sodium dodecyl sulphate	WBW_{sw}: Whole-body weight measured at the saltwater site
SGR: Specific growth rate	W_{end}: End weight (g)
siRNAs: Small interfering RNAs	WGD: Whole genome duplication
SDY: Sex-determining region Y gene	W_{start}: Start weight (g)
sm: Siemens per meter	WW_{waste}: Wet weight (g) of uneaten feed
small ncRNA: Small non-coding RNA	

Terms and Definitions

A matrix: Genetic relationship between individuals based on pedigree information.

Adipose fin: Small fleshy fin, located between dorsal fin and tail.

Alkalinity: Measurement of water's capacity to resist acidification.

Allotetraploidy: Whole genome duplication arising from the fusion between two species.

Always-methylated: Average methylation proportion of 1.

Always-unmethylated: Average methylation proportion of 0.

Anadromous: Life cycles which include migration from freshwater nursery and breeding environments to saltwater feeding grounds.

Autotetraploidy: Whole genome duplication arising from spontaneous doubling of a single genome

Biofilters: A biological filter populated with microorganisms capable reducing ammonia toxicity.

Bi-/multi- variate models: Quantitative genetic model where two or more traits are co-currently fit, and parameters estimated.

Box-cox transformation: Mathematical transformation which increases normality of data and helps stabilize variance.

Break-even correlation: Under the assumption costs of running a single larger breeding program is the same as two smaller environment-specific ones, this is the intersection of genetic correlations when genetic gain of the two programs is equal (Mulder *et al.*, 2006; Sae-Lim *et al.*, 2016).

Broodstock Conditioning: Exposure of broodstock to specific environments/environmental conditions to favourably shape the epigenome of offspring.

Broodstock/Nucleus populations: Adult fish population who undergo selection and supply genetic material to farms for commercial production.

Carbonate buffering system: Acid-base homeostatic reaction that regulates pH level.

Cell differentiation: Process by which cells develop specificity via differential gene expression patterns.

Chromatin: Complex of DNA and histone proteins organised as nucleosomes, packaging DNA in the nucleus.

Circadian: Processes that follow an approximate 24-hour periodicity. While driven by an internal, endogenous, pacemaker they can be influenced by environmental information.

Circannual: Processes that follow an approximate 1-year periodicity. While driven by an internal, endogenous, pacemaker they can be influenced by environmental information.

Cis-regulatory: Non-coding regions of DNA that regulate transcription of neighbouring or nearby genes.

Common family effects: In quantitative genetics, these are effects that influence phenotypic variance and derive from period of separate familial rearing.

Condition factor: Allometric measurement of weight and length, indicating 'chubbiness'.

CpG site: Where a cytosine is in a 5'-cytosine-phosphate-guanine-3' sequence.

CpG Islands: DNA regions dense in CpG sites.

Dam: Female parent.

Days in SW: The number of days between the date of SW transfer and SW sampling.

Degree days: $T^{\circ}C * \text{number of days}$.

De-methylation: Active or passive loss of cytosine methylation.

De-smoltification: Process of losing some smolt characteristics if downstream migration does not occur.

Developmental programming: Exposure of eggs, larvae, or juveniles to specific environments/environmental variables to favourably shape the epigenome.

Differently methylated cytosine (DMC): Differential methylation relative to control, filtered based on both false discovery rate and p-value.

Divalent cations: Positively charged ions with a +2 charge.

Electrochemical gradient: Combined gradient of different solutes (chemical) and charges (electro) across a membrane, driving the movement of ions.

Endogenous: Originating from within an individual.

Endothelial cells: Specialised cells which form the endothelium, a single cell layer lining blood and lymphatic vessels.

Epialleles: Heritable differences in gene expression that are derived from homogenous DNA sequences via alternative epigenetic sites.

Epigenetic miss-match: Where epigenetic patterns are mal-adaptive because environmental exposure is too dissimilar between early and later life, or generations, for example.

Epigenetic priming: Exposure to an environmental stimulus that helps guide

epigenetic programming and correct development or generates an epigenetic memory which better allows the individual to respond to the same stimulus later in life.

Epigenetics: Chromosome-bound, mitotically or meiotically heritable changes to gene expression, independent of DNA changes itself (Deans and Maggert, 2015; Best *et al.*, 2018; Eirin-Lopez and Putnam, 2019).

Epigenome: Genome wide pattern of epigenetic signatures or modifications

Epimutation: Heritable alteration of gene expression independent of changes to the DNA sequence itself.

Euchromatin: Loosely packed chromatin state, which is permissive for gene transcription.

Exogenous: Originating externally to the self.

Exon: Intragenic region that encodes proteins.

Extra FW days: The number of days between the date of freshwater sampling and saltwater transfer.

F1 generation: The first generation produced from crossing parents.

F2 generation: The offspring produced from crossing of the F1 generation, themselves the first generation produced from crossed parents.

Factor analytic structures: Used to simplify complex data to fewer underlying factors which explain observed patterns.

Feed conversion efficiency: How efficiently feed is converted into biomass.

Floating net-pens: Aquaculture rearing environment where large net like cages float within water bodies.

Flow-through system: Aquaculture rearing environment, often land-based, where water is continuously circulated through tanks or channels.

Gametogenesis: Process of producing germ, or sex, cells.

Ganglion cells: Neurons located in the retina which transmit visual information.

Genotype: An individual's specific set of genetic variants across the genome.

Grading: Sized based sorting of fish.

Grilse: Salmon which mature after a single winter at sea.

H Matrix: Genetic relationship between individuals, combining both SNP (GRM) and pedigree (A matrix) information, as defined in Legarra *et al* (2014).

Hardness: Measurement of the concentration of dissolved minerals in water.

Heterochromatin: Densely packed chromatin state, which is inhibitory to gene transcription.

Hyper-osmotic: A higher concentration of solutes compared to the surroundings.

Hyper-methylated: Higher average proportion methylation relative to control conditions.

Hypo-osmotic: A lower concentration of solutes compared to the surroundings.

Hypo-methylated: Lower average proportion methylation relative to control conditions.

Imprinting genes: Genes whose expression is inherited in a parent of origin manner, such that the copy from one parent is silenced.

Intergenic: DNA regions in between gene bodies.

Intron: Intragenic region that is not expressed.

Iso-osmotic: Solutions which have the same concentration of solutes/the same osmotic pressure.

Lambda (λ): Defined as $\text{median}(\chi^2) / \text{expected median}(\chi^2)$, where values greater than one indicate test statistic inflation possible population stratification/overdispersion (Yang *et al.*, 2011).

Latent common effects: In factor analytic approaches, this represents underlying genetic factors which explain similarity and observed correlation amongst traits.

Linkage disequilibrium: Non-random association of alleles.

Lipophilic: Lipid dissolvable.

Macroenvironment: A broader/larger environment or environmental variables

Mal-adaptive programming: Where early environmental exposure shapes the epigenome such that it is less suited to future environments or environmental exposure.

Messenger RNA: Produced during transcription and provides the template for the ribosome to create proteins.

Methylome: DNA methylation patterns across the whole genome.

Methyl Quantitative Trait Loci: Genetic loci associated with variation in DNA methylation.

Microenvironmental sensitivity: An individual's ability to buffer against unknown biotic and abiotic disturbances within a single macroenvironment, as well as developmental or endogenous disturbances (Sonesson, Ødegård and Rønnegård, 2013).

Multiplier stations: Sites which grow their own broodstock from genetic material

supplied from central breeding programs and supply genetic material to local farms for grow-out.

'n' base: Added to sequences when missing base is present.

Nephrocalcinosis: Calcium salt depositions in renal tissue.

Nested mating: Mating of one male to multiple females, producing sets of full and half sib families.

Neuroendocrine: System and tissues of both nervous and endocrine system.

Neurotransmitter: Chemical messengers that signal across a synapse between neurons.

Nucleosome: Fundamental unit of chromatin, consisting of negatively charged DNA wrapped around an octamer of positively charged histone proteins.

Ohnologs: Gene duplicates originating from a whole genome duplication event

Ova: eggs/female reproductive cells.

Para-cellular: Movement of substances between cells.

Parr: Predominant freshwater life stage of salmon.

Partial pressure: In a mixture of gasses, the pressure a single gas would exert if it occupied the same space as the mixture.

Polynomial function: Mathematical expression that includes terms which raises a valuable (x) to a positive integer exponent (e.g. x^3). The order of the function is the highest value exponent.

Polynomial coefficient: The positive multiplicative integer of a term in a polynomial function (e.g. the 2 in $2x^3$).

Polyploid: Organism which has more than one copy of its genome

Phenotype: The combination of observable characteristics belonging to an individual.

Phenotypic

plasticity/macroenvironmental

sensitivity: The ability of a single genotype to produce multiple phenotypes based on environmental exposure.

Photoperiod: Relative length of day light hours.

Photoreceptors: Specialized cells capable of phototransduction.

Phred value: Represents the confidence in the assessment of base calling performed by a sequencer, where high scores demonstrate greater confidence.

Pineal gland: Melatonin secreting gland/tissue.

Post-smolt: Initial life stage of salmon after migration to sea.

Primordial germ cells: Arise early in embryogenesis and are precursors to germ/sex cells.

Promoter: Site of transcription initiation, located up-stream of intragenic regions of a gene, containing the necessary machinery and specific binding sequences for RNA polymerase II.

Pseudo-smolts: Smolts with poor saltwater performance and survival.

Putative DMC: Differentially methylated cytosines filtered based only on their significance (P-value).

Quantitative Trait Loci: Genetic loci associated with variation in a phenotypic trait.

Reaction norms: Pattern of phenotypic expression of a genotype across environments or along an environmental gradient.

Redds: Gravel beds in natal rivers returning adult Atlantic salmon release eggs into for fertilization.

Rediploidization: The process of returning from a polyploid to diploid state

Re-ranking: Change in the relative family performance between environments or along an environmental gradient.

Robustness: The realisation of high production potentials for a genotype across a wide range of environmental conditions, all whilst being resistant to environmental stressors.

Scaling effect (GxE): Heterogeneous additive genetic variance between environments or along an environmental gradient.

Scaling effect (variance): Changes in trait variance as a function of the mean.

Single-Step Genomic Evaluation: Analysis of pedigree, genotype and phenotypic data in a single analytical step.

Sire: Male parent.

Smolt: Life stage of Atlantic salmon adapted for downstream migration to saltwater.

Smoltification/Parr-smolt transformation: Process of morphological, physiological and behavioural adaptations to saltwater environment acquired during parr to smolt development.

SNP array: Type of DNA microarray to genotype individuals and identify SNPs within a population.

Somatic cells: Tissue (non-sex) cells.

Specific effects: In factor analysis, this represents the environmental influence over traits.

Synaptic junction/synapse: Point where two nerve cells meet, a gap between which signals are sent.

(Sib-) Testing station: Progeny of broodstock is tested under conditions at farm sites.

Totipotency: The ability of a cell to differentiate and specialise into any cell type.

Trans-cellular: Movement of substances through/across cells and membranes.

Transcription start site: Site of transcription initiation, located downstream of the core promoter.

Transcription terminations site: The site where transcription is stopped and synthesised mRNA sequence released.

Transcription: Process by which DNA is read and corresponding mRNA strand produced by the action of RNA polymerase II.

Transgenerational epigenetic inheritance: In fish, epigenomic signature of gametes passed onto at least the F2 generation.

Translation: Reading of mRNA strands by ribosomes to produce proteins.

Trans-regulatory: DNA sequences which regulate gene expression of distal genes.

Variable-methylation: average methylation less than 1 and greater than 0.

Species list

Clupea harengus, Atlantic herring
Crassostrea angulate, Portuguese oyster
Cyclopterus lumpus, Lumpfish
Cyprinus carpio, Eurasian carp
Danio rerio, Zebrafish
Gasterosteus aculeatus, Three-spine stickleback
Larimichthys crocea, Large yellow croaker
Penaeus (Litopenaeus) vannamei, Whiteleg shrimp
Oncorhynchus kisutch, Coho salmon
Oncorhynchus mykiss, Rainbow trout
Oreochromis niloticus, Nile Tilapia
Penaeus japonicus, Kuruma prawn or Japanese tiger prawn
Salmo salar, Atlantic salmon
Salvelinus alpinus, Artic charr

Chapter 1-General introduction

Aquaculture production has increased over the 21st century fulfilling the growing demand not met by capture fisheries (Clarke and Bostock, 2017; FAO, 2020). Notably, the percentage of aquatic animal production derived from aquaculture recently surpassed capture fisheries for the first time (FAO, 2024b). Globally, Atlantic salmon, *Salmo salar* L. 1758, is an economically important species, in particular due to its contribution in terms of monetary value rather than purely tonnage produced (FAO, 2023, 2024a). In the UK, Atlantic salmon dominates the industry, where the vast predominance of production occurs in Scotland. On average 170,421 tonnes of salmon, valuing £787,837,562, were produced from 2013-2018, making the UK the third largest producer of Atlantic salmon globally after Norway and Chile (Regan *et al.*, 2021; FAO, 2023).

This industry expansion has been, in part, enabled by progress achieved by programs of selective breeding as well as technological advancements (Gjedrem, 2000; Ellis *et al.*, 2016). While extensive research into the genetic parameters of key traits (e.g. Powell *et al.*, 2008; Garber *et al.*, 2019) has allowed commercial production to improve and regulate phenotypes, this is also dependent on the environment (Falconer, 1952; Falconer and Mackay, 1996). In a commercial setting, loss of control over phenotypic expression can be costly in terms of its economic value, processing costs, and husbandry, as well as having implications for programs of selection (Khaw *et al.*, 2016; Marjanovic *et al.*, 2016; Berghof, Poppe and Mulder, 2019). Importantly, however, there is now increasing interest in the use of recirculating aquaculture systems (RAS) over other culture systems due to the advantages they offer (including biosecurity, mitigation of environmental impacts, or husbandry control, amongst others) despite significant economic investment (Bergheim *et al.*, 2009; Ebeling and Timmons, 2012). Therefore, understanding how the use of this novel environment could influence phenotypic expression in selected populations is of keen interest.

Existing understanding of Atlantic salmon development and commercial production is presented here, as well as exploring information on the role the environment plays in this via both genotype-environment interactions (GxE) and epigenetics mechanisms.

1.1. Atlantic salmon life cycle and biology

Atlantic salmon is a species of the *Salmonidae* family of ray-finned fish inhabiting regions across the North Atlantic Ocean on both the European and North American coasts (Klemetsen *et al.*, 2003; FAO *et al.*, 2009). Importantly, their life cycle involves several life stages with distinct phenotypic types and habits. This falls in line with their anadromous migration patterns where they leave freshwater (FW) natal rivers after juvenile development for saltwater (SW) feeding grounds, after which they undergo maturation and migrate back to natal rivers to spawn and reproduce (Figure 1-1).

Specifically, life cycles typically start in winter, where retuning mature adults spawn in natal FW rivers. These fish release eggs to be fertilized in gravel beds, termed 'redds'. Eggs then go through embryonic developmental stages before hatching in the spring. Commercially, hatching has been recorded between 480-to-520-degree days ($T^{\circ}\text{C} \times \text{number of days}$) after fertilization (Benchmark Genetics, 2022). With typical larval stages missing, fish emerge as alevins (recorded between 850-to-920-degree days after fertilisation) and live off endogenous yolk sacks (Gorodilov, 1996; Mobley *et al.*, 2021; Benchmark Genetics, 2022). Once independently feeding as fry, juvenile fish stay in these FW rivers feeding and grow through to the parr developmental stage. The length of time fish remain in FW can vary from approximately 1-3 years during which they may undergo smoltification, also known as smolting or the parr smolt transformation (PST), during late spring to early summer (McCormick *et al.*, 1998; FAO *et al.*, 2009; Good and Davidson, 2016; Mobley *et al.*, 2021). Importantly the PST develops the physiological, behavioural, and morphological adaptations that prepare fish for their downstream migration and SW entry as smolts (Sheridan, 1989; McCormick *et al.*, 1998; Stefansson *et al.*, 2008; Seear *et al.*, 2010; McCormick, 2012; Gillard *et al.*, 2018).

Once at sea, post-smolts travel to key northern feeding grounds, such as the west (north American populations) and east (northern European populations) of Greenland, where they remain as immature adults for multiple years (Klemetsen *et al.*, 2003; Ounsley *et al.*, 2019; Almodóvar *et al.*, 2020). When maturation is triggered, fish develop secondary sexual characteristics, cease feeding, and return to natal rivers for spawning (Hansen and Quinn, 1998). Unlike some salmonids, Atlantic salmon are capable of multiple migrations between

FW and SW, potentially reproducing multiple times (Klemetsen *et al.*, 2003; Good and Davidson, 2016) (Figure 1-1).

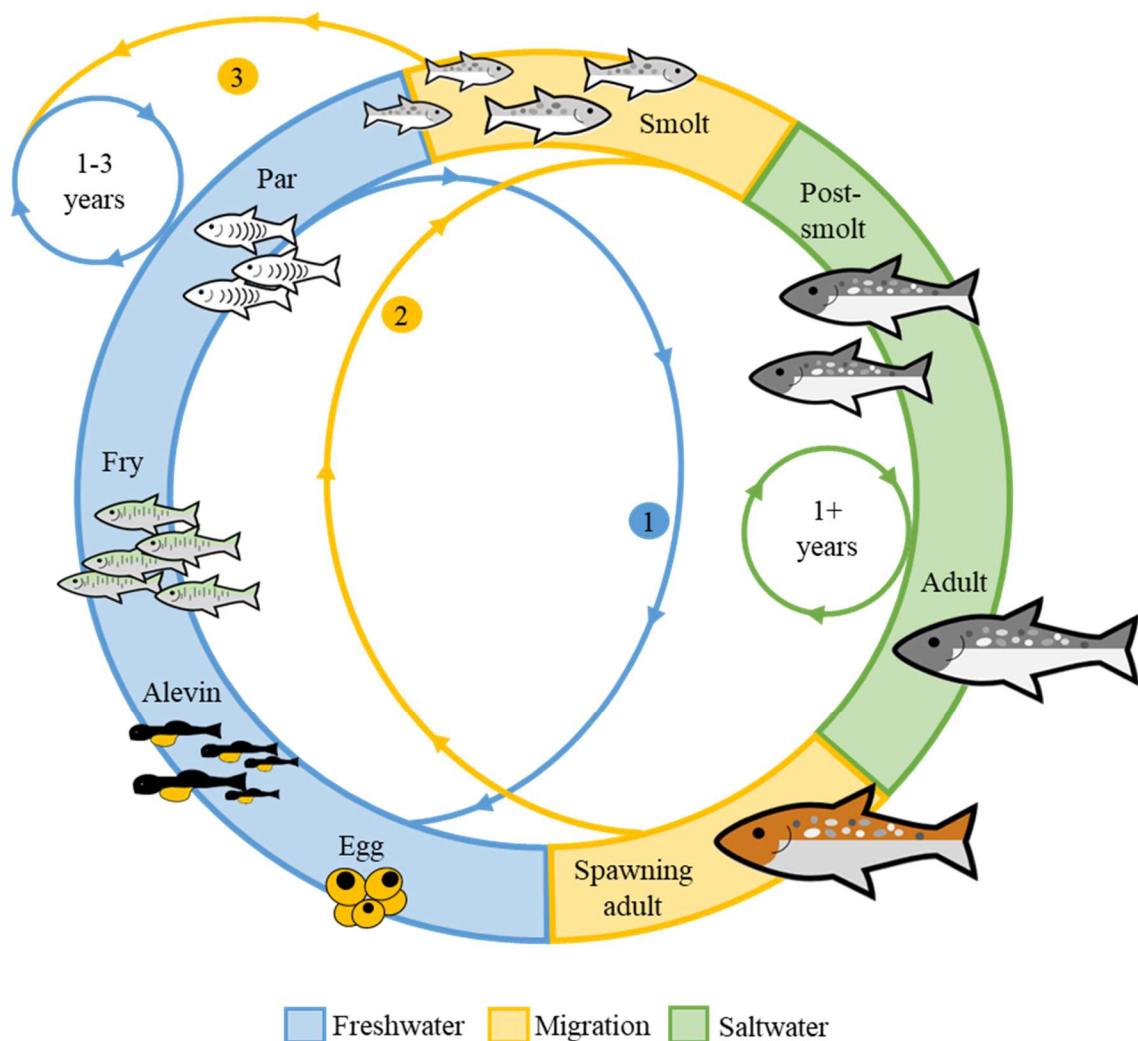


Figure 1-1 Atlantic salmon life cycle. Pathway one represents premature maturation as parr, pathway two repeat migration of adults, and pathway three is 'de-smoltification'. Inspired by (Mobley *et al.*, 2021).

Within this life cycle, however, there is variability (Klemetsen *et al.*, 2003). FW residency can vary, relating to size and growth rate (McCormick *et al.*, 2007; McCormick, 2012). Specifically, parr show a bi-modal size distribution containing an upper and lower mode. Those in the upper mode who reach a size threshold (~12cm winter size) are destined for smoltification and continue to feed through winter. Those in the lower mode instead remain as parr for at least a further year (McCormick *et al.*, 2007; Stefansson *et al.*, 2008). Maturation timing is also variable (Good and Davidson, 2016). While some adult salmon mature after one winter at sea (termed grilse) others spend multiple years in SW (Good and Davidson, 2016). Alternatively, maturation need not occur at sea but can happen prematurely as parr (Klemetsen *et al.*, 2003)

(Figure 1-1). Overall, the decision to undergo maturation is genetically, epigenetically, and environmentally regulated (Nichols *et al.*, 2008; Morán and Pérez-Figueroa, 2011; Good and Davidson, 2016; Mobley *et al.*, 2021).

1.1.1. The smolt phenotype

A key stage of the Atlantic salmon FW phase is smoltification. This transition usually occurs in spring and comprises the development of phenotypic traits priming fish for SW entry (Stefansson *et al.*, 2008). Morphological changes include increased size, loss of parr marks and body silvering, fin margins darkening, and reduced condition factor through increased linear compared to body growth (McCormick *et al.*, 1998; McCormick, 2012). Behaviourally there is a loss of aggressive territorialism, proximity to riverbeds, and positive rheotaxis. Instead, smolts show increased buoyancy, active movement downstream while swimming into areas of high velocity, as well as the formation of aggregations once at sea (McCormick *et al.*, 1998; Stefansson *et al.*, 2008). Physiologically, total lipid (primarily triacylglycerol) content decreases in response to increased catabolism of lipid depots (adipose tissue, dark muscle, and liver) as an energy source, as well as a reduction in their synthesis (Sheridan, 1989). Secondly, the composition of bodily lipids also shifts, in line with the adoption of SW diets (Sheridan, 1989; Stefansson *et al.*, 2008; Gillard *et al.*, 2018). Furthermore, oxygen consumption and transport increase, with the synthesis of additional haemoglobin isoforms (Stefansson *et al.*, 2008).

A key challenge of SW entry, however, is the transition from a hypo- to hyper-osmotic environment, causing smolts to lose water via osmosis and diffusively gain ions (McCormick, 2012; Morera *et al.*, 2015). To counteract the effect of these salinity differences, the behaviour and physiology of salmon's osmoregulatory tissues (gills, gut and kidneys) change. While in FW ionocytes of the gill epithelium uptake sodium (Na^+) and chlorine (Cl^-), once in SW these ions are actively extruded via electrochemical gradients generated by the movement of potassium ions (K^+) and various membrane channels (Evans, Piermarini and Potts, 1999; Hirose *et al.*, 2003; Hwang, Lee and Lin, 2011). Additionally, transcriptional changes of channel pore proteins, expression of specific isoforms, and changes to location size and number of ionocytes occur (Evans, Piermarini and Potts, 1999; McCormick, 2012). Behaviourally, smolts actively drink SW to counteract what is passively lost to their surroundings. To enable both para- and trans-cellular water reabsorption from ingested SW,

Na⁺ and Cl⁻ are absorbed across intestinal epithelial tissues, similarly powered by electrochemical gradients and the movement of K⁺ (McCormick, 2012; Whittamore, 2012). Any absorbed salts are then extruded across the gills and excess divalent cations are lost in small amounts of iso-osmotic urine produced by the kidneys (McCormick, 2012; Sundh *et al.*, 2014).

1.1.2.Regulation and onset of smoltification

Development of this smolt phenotype is regulated by several neuroendocrine pathways via the light-brain-pituitary axis. Its onset is associated with spring surges in growth hormone (GH), cortisol, insulin-like growth factor 1 (IGF-1) and thyroid hormones (triiodothyronine, T3 and thyroxine, T4) (McCormick *et al.*, 2002; Stefansson *et al.*, 2008; McCormick, 2012), which are responsible for the development of specific smolt characteristics. Prolactin and sex hormones, by contrast, have an inhibitory effect (Figure 1-2) (McCormick *et al.*, 2007; McCormick, 2012). Importantly, for correct development the action of these multiple pathways and hormones requires synchronisation. If this fails to happen and the pathways instead become uncoupled, 'pseudo-smolts', with poor SW performance and survival, maybe produced (Stefansson *et al.*, 2008). Furthermore, the onset of smoltification needs to be triggered at the correct time of year when ecological conditions encourage the best chances in the marine environment. If a miss-match occurs between these physiological and ecological windows, smolts may not migrate downstream and instead undergo 'de-smoltification' (Figure 1-1) where they lose some of their smolt characteristics (McCormick *et al.*, 1998; Stefansson *et al.*, 2008; McCormick, 2012).

While development of SW adaption is under genetic control with certain loci (Nichols *et al.*, 2008; Le Bras *et al.*, 2011; Martínez, Garza and Pearse, 2011; Norman *et al.*, 2012; Hale *et al.*, 2013) and transcriptional variation (Seear *et al.*, 2010; Robertson and McCormick, 2012) associated with SW performance and smoltification, its timing is likely regulated by an interaction between exogenous environmental stimuli and some aspect of an endogenous circannual rhythm (McCormick, Moriyama and Björnsson, 2000). The key exogenous cue appears to be light. Specifically, it is the relative day length or photoperiod that principally plays a role in the onset of smoltification (McCormick *et al.*, 2002). Importantly, photoperiod length changes throughout the year due to the earth's axis, rotation on its axis, and revolution around the sun.

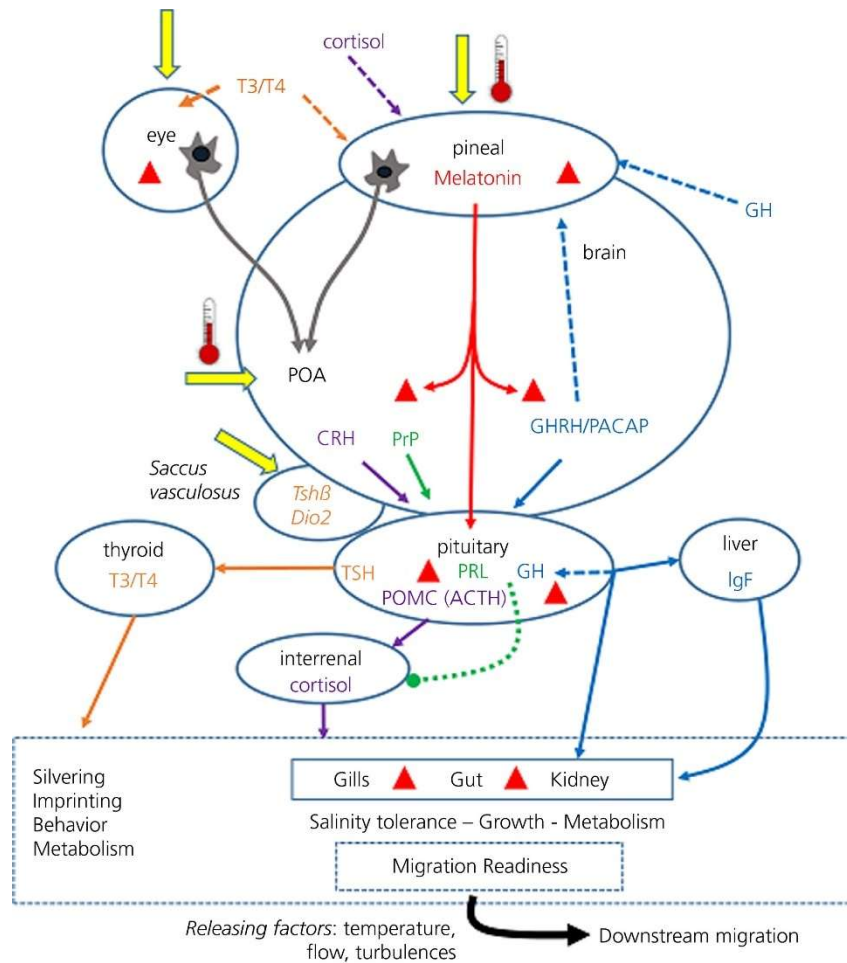


Figure 1-2 From Nisembaum *et al.* (2021): Inter-relationships between the photoperiodic and smoltification systems. In this: Δ : melatonin receptors; yellow arrows: sites of light perception; thermometers: sites of temperature perception. Acronyms are as follows: ACTH adrenocorticotrophic hormone; CRH, corticotrophin-releasing hormone; Dio2, deiodinase 2; GH, growth hormone; GHRH, growth hormone-releasing hormone; PACAP, pituitary adenylate cyclase-activating polypeptide; POA, preoptic area; POMC, pro-opiomelanocortin; PRL, prolactin; PrP, prolactin-releasing peptide; T3/T4, thyroid hormones; TSH, thyroid-stimulating hormone. Arrows with solid lines indicated proven direct effects of a hormone, and dashed lines indicated suggested but not yet confirmed direct effects. The line endings indicate either activation (arrow end) or inhibition (circle end) effects. The colours of the arrows correspond to the hormones written in the same colours, which are grouped according to their endocrine pathway and their roles in physiology, morphology, metabolism and/or behaviour.

Specifically, the earth rotates on its axis over approximate 24-hour period, generating periods of dark (when facing away from the sun) and light (when facing the sun). However, the earth's axis is tilted and the revolution around the sun an elliptical shape. This generates (1) a variable number of daylight hours throughout the year, and (2) different lengths of daylight across the globe (Falcón *et al.*, 2010; Migaud, Davie and Taylor, 2010). Notably, while at the equator the number of light and dark hours remain relatively equal and stable through the year, as latitudes increase the magnitude by which these changes increases, as well as the direction of change being opposite in northern compared to southern hemispheres. Considering this, it is generally accepted that for parr to undergo smoltification, exposure to both winter short days

and increasing daylight hours of spring following the winter solstice are needed (McCormick, Moriyama and Björnsson, 2000; McCormick *et al.*, 2002; Stefansson *et al.*, 2007), though a threshold day length in spring has also been suggested (Strand, Hazlerigg and Jørgensen, 2018). Furthermore, there is also evidence of physiological changes before the winter solstice that allow smolting parr to be responsive to increasing spring photoperiods (Ebbesson *et al.*, 2007; McCormick *et al.*, 2007).

Secondary to changing day length, temperature also plays a role in regulating smoltification and while it is unable to trigger smoltification itself, it acts to fine-tune its timing and developmental pattern (McCormick *et al.*, 2002). Specifically, increased temperature can advance the onset of smoltification and is thought to hasten the acquisition and subsequent loss of smolt characteristics, narrowing the physiological smolt window (McCormick, 2012). In contrast, low temperature appears inhibitory to the development of smolt characteristics and limits the ability of light to trigger smolting via the damping of hormonal surges (McCormick, Moriyama and Björnsson, 2000; McCormick *et al.*, 2002). Considering this, it has been suggested that it is the cumulative temperature experienced, conceptualized as degree days, that affects smoltification (Sigholt *et al.*, 1995; McCormick *et al.*, 2002).

For photoperiod, and to a lesser degree temperature, to regulate the timing and progression of smoltification it needs communicating internally so it can direct the characteristic synthesis and release of hormones, as described above. In mammals, the photoperiod cues are translated into physiological processes through the photoneuroendocrine system (PNES). The PNES comprises photic information communicated via retinal photoreceptors to the suprachiasmatic nucleus (SCN), the location of a master circadian clock, and the pineal gland which releases melatonin at night conferring information of day length (Migaud *et al.*, 2007; Migaud, Davie and Taylor, 2010). Importantly, in mammals, there is a circadian rhythm in melatonin synthesis, release and concentration that is maintained by this master clock which will persist in the absence of environmental information over approximately 24 hours. The reception of photic information can, however, entrain this cycle, accurately aligning it to the external environment. As day length fluctuates throughout the year, changes in the length of melatonin release can inform on both circadian and circannual (variation over a year) rhythms (Migaud, Davie and Taylor, 2010).

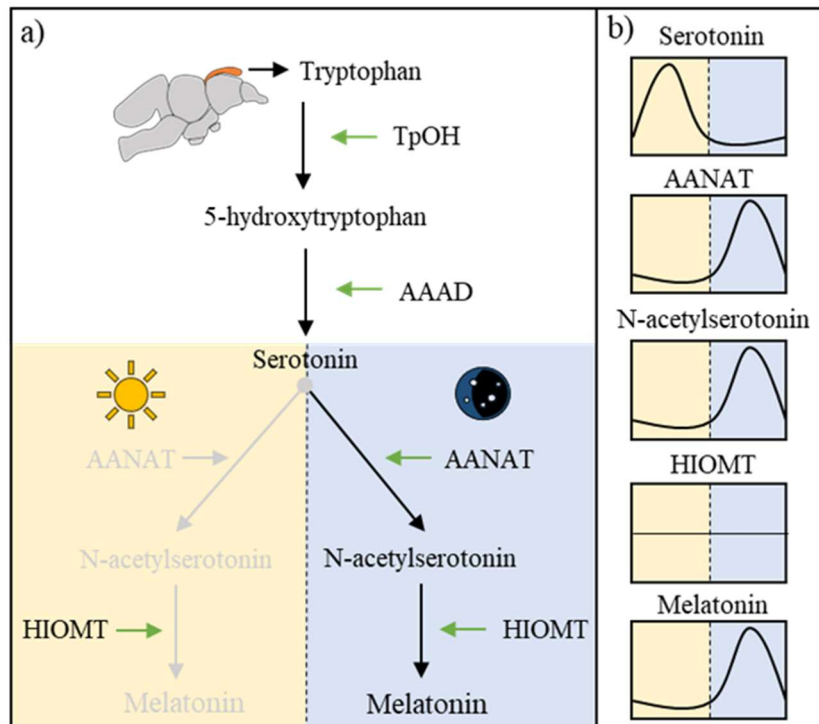


Figure 1-3 Biosynthesis of pineal melatonin (panel a) showing its nocturnal production and light inhibition. Changes in key proteins /enzymes abundance shown in panel b. Orange brain region indicates pineal gland. TpOH is Tryptophan hydroxylase, AAAD is Aromatic amino acid decarboxylase, AANAT is Arylalkylamine N acetyltransferase, and HIOMT is hydroxyindole-O-methyltransferase. Green arrow indicates enzymatic action, and greyed out sections indicates their concentration drops under daylight. Based on information in Migaud, Davie and Taylor (2010), Falcón *et al.* (2010) and figure produced in Nisembaum *et al.* (2021).

PNES of teleost fish is less well understood and studied but has been found to show two large differences from the mammalian models: (1) that the pineal gland is directly photosensitive and (2) there is no centralized master circadian clock (Migaud, Davie and Taylor, 2010). Instead, in most teleosts the PNES is organized as a set of interconnected circadian clocks located throughout tissues and/or organs along with pineal photoreceptors which biosynthesize melatonin over a circadian cycle in direct response to light dark cycles (Falcón *et al.*, 2010). In brief, when pineal photoreceptors are exposed to light via the cranial window, it causes the post-translational inhibition of arylalkylamin N acetyltransferase (AANAT). Importantly AANAT plays a role in the catalysis of melatonin from serotonin, driving nocturnal rise and daylight decreases in melatonin (Falcón *et al.*, 2010; Migaud, Davie and Taylor, 2010; Nisembaum *et al.*, 2021) (Figure 3-1). While in some teleost species there is evidence of an endogenous clock within the pineal gland that maintains melatonin production independent of light exposure, this is not present in salmonids (Migaud *et al.*, 2007; Nisembaum *et al.*, 2021). Instead, this has either been lost or become decoupled from pineal

melatonin biosynthesis, which is now only directly regulated by light exposure in an on/off fashion (Davie, Minghetti and Migaud, 2009; McStay *et al.*, 2014) (Figure 1-3).

As melatonin is lipophilic it readily leaves the pineal gland. As a consequence circulating levels of melatonin respond to and closely match the prevailing photoperiod (Migaud, Davie and Taylor, 2010). Across both central and peripheral tissues, melatonin receptors have been found, including the preoptic area (POA) of the brain, liver, gills and importantly the pituitary gland which is involved in the downstream release of hormones associated with smoltification (Nisembaum *et al.*, 2021) (Figure 1-2). In addition to this hormonal signalling, pineal photoreceptors release excitatory neurotransmitters at the synaptic junction with secondary or ganglion cells at night. These latter cells then convey electrical information to brain centres and as with melatonin production, is inhibited by light. While many brain regions are targets, the key regions where the majority of nerve endings project into include the POA, which integrates photic information from both the retina and pineal. How the action of these neural and hormonal signalling relates to and helps regulate smoltification is not fully understood (Nisembaum *et al.*, 2021). However, it does appear to operate within the light-brain-pituitary axis to direct the release of hormones driving the development of the smolt phenotype (Figure 1-2).

1.2. Atlantic salmon aquaculture

1.2.1. Industry structure and production cycle

One of the earlier species to undergo commercialisation, Atlantic salmon production began in the early 1970s in Norway and has now expanded within the species natural range (e.g. Ireland, UK, Faroe Islands and Iceland) and beyond (e.g. Chile, North America and Australia) (Bergheim *et al.*, 2009; FAO, 2020; Verbyla, Kube and Evans, 2022). As an industry its growth can be characterised by a period of rapid expansion in productivity and farm number, which has slowed since the late 1990s and early 2000s, stabilising post 2014 (Kolarevic *et al.*, 2014; Murray and Munro, 2018). During this time there has been progressive consolidation of the industry, where the number of farms has decreased while their size and productivity have increased. The industry is now dominated by a few, mainly Norwegian, companies (Ellis *et al.*, 2016; Murray and Munro, 2018).

Production cycles include several distinct environments, starting with a nucleus population of the breeding individuals or broodstock who are subject to selection. These fish are not harvested but supply the genetic material (either smolts or ova) for farms to grow-out fish for commercial production (Ellis *et al.*, 2016). While some countries (e.g. Chile, Lhorente *et al.* (2019)) have their own breeding populations, many farms are supplied with ova by large international companies from centralised nucleus populations or more local multiplier stations (Ellis *et al.*, 2016; Houston and Macqueen, 2019; Lhorente *et al.*, 2019; Verbyla, Kube and Evans, 2022). For example, the proportion of foreign ova supplying Scottish farms has increased since 2006 to become the predominant source of genetic material (Ellis *et al.*, 2016). Recently, however, there has been a ban on Norwegian exports to Scotland, meaning alternative or endogenous populations have taken over this supply (Holmyard, 2020). Furthermore, as an anadromous species, production requires FW hatchery and nursery environments before smoltification, as well as transfer to SW sites for grow-out. Fish are typically harvested at ~3-5kg, prior to maturation and development of undesirable secondary sexual characteristics (Ellis *et al.*, 2016).

1.2.2. Programs of selective breeding and genomic analysis

In comparison to other aquacultural species, programs of selective breeding for Atlantic salmon are more established, also having begun in the 1970s (Teletchea and Fontaine, 2014). These programs have chosen traits of economic value and have substantially contributed to increases in the production and performance of *S. salar*. Breeding goals include characteristics which (1) reduce production cost (e.g., growth rate, feed conversion efficiency and age of sexual maturity), (2) relate to fish welfare and functionality (e.g., saltwater survival, and disease resistance), and (3) relate to produce quality (e.g., fillet pigment and fat content) (Gjedrem, 2000; Gjerde *et al.*, 2007), with interest in resilience and robustness traits increasing over recent years (Berghof, Poppe and Mulder, 2019).

Over this period, a range of genomic tools have been developed. This includes the relatively simple family-based selection, where breeding candidates are closely related to fish with desirable characteristics (Houston and Macqueen, 2019). However, this does not take advantage of the genetic variation within families. Importantly, many key traits (as described above) are quantitative and show a continuous gaussian distribution throughout a population

(Falconer and Mackay, 1996; Barton, Etheridge and Véber, 2017). Based on this premise more advanced tools have been developed which make use of both phenotypic and genetic data. An example is marker assisted selection (MAS) in which genomic regions associated with good performance can be identified (i.e. quantitative trait loci, QTL) and breeding candidates with such regions selected (Houston and Macqueen, 2019).

Similarly, genomic selection had been developed enabling the estimation of an individual's genetic merit (Gjerde et al., 2007; Huston, 2017; Huston and Macqueen, 2019). In this, breeding is based on partitioning the phenotypic variance (V_p) of multi-generational populations into different components such as genetic (additive (V_g) and dominance (V_d)) and environmental (V_e) variance, though other components can be included (e.g. maternal effects) (Falconer and Mackay, 1996). Specifically, phenotypic variance can be decomposed as:

$$V_p = V_g + V_d + V_e \dots \quad \text{Eq. 1-1}$$

Importantly, these components are central to estimating key parameters of interest: narrow sense heritability (h^2), estimated breeding values (EBV), and genetic correlations between traits (r_g) (Falconer and Mackay, 1996; Barton, Etheridge and Véber, 2017). In brief, h^2 is the proportion of phenotypic variance explained by additive genetic variance, EBV represents an individual's genetic merit and is used in selecting reproducing individuals, and r_g indicates the correlated change expected in non-selected traits (Falconer and Mackay, 1996; Gjerde et al., 2000; Wilson et al., 2010). Of note, the presence and relative size of additive genetic variance indicates the potential to selectively alter the trait of interest (Gjedrem, 2000; Garber et al., 2019).

In practice, variance components are estimated from quantitative genetic models (QGM), such as:

$$\mathbf{y} = \boldsymbol{\mu} + \mathbf{X}\mathbf{b} + \mathbf{Z}\mathbf{u} + \mathbf{e} \quad \text{Eq. 1-2}$$

in which $\mathbf{y}$ would be a vector of phenotypic values, $\boldsymbol{\mu}$ the overall population mean, $\mathbf{X}$ design matrix linking individuals to the vector of fixed effects $\mathbf{b}$, $\mathbf{Z}$ the design matrix linking individuals to vector of random effects $\mathbf{u}$, and $\mathbf{e}$ a vector of residual effects (Falconer and Mackay, 1996; Barton, Etheridge and Véber, 2017; Garber et al., 2019).

For key economic traits significant genetic variation and low-to-moderate heritabilities have been identified, suggesting the opportunity for selective breeding (Gjerde *et al.*, 2007; Powell *et al.*, 2008; Garber *et al.*, 2019). Due to salmon's high fecundity, phenotypic variation, and moderate generation interval (~4 years), high genetic gains were expected and have been realised for some traits (e.g. 10-15% per generation for growth rate) (Gjedrem, 2000). Genetic correlations, often measured between body weight and secondary traits, appear mostly low or favourable, indicating that selection for one trait either would not affect a second trait or will alter it in a desired direction, respectively (Gjedrem, 2000). Estimates of genetic parameters are, however, variable. Traits are defined and measured differently across studies, and it has been suggested that the environmental and genetic background of breeding populations causes further variability in estimates, emphasizing that parameters are often population specific (Powell *et al.*, 2008; Garber *et al.*, 2019).

It should be noted that Atlantic salmon have undergone several whole genome duplication (WGD) events. WGDs produce an additional copy of every gene, termed ohnologs, either by fusion or hybridisation between two species (allotetraploidy) or spontaneous doubling of one (autotetraploidy) (Van de Peer, Maere and Meyer, 2009; Berthelot *et al.*, 2014; Warren *et al.*, 2014). Specifically, Salmonids (those belonging to the *Salmonidae* family: Salmon, Trout, Charr, Freshwater whitefish and Graylings) have undergone four rounds of autotetraploidy WGD, including two common to all vertebrates (1R and 2R), a third shared by teleosts (Teleost specific 3R, Ts3R), and a fourth (Salmon specific 4R, Ss4R) occurring in a shared *Salmonidae* ancestor approximately 88 to 103 million years ago (Macqueen and Johnston, 2014). While such autotetraploidy events results in genetic redundancy which facilitates genomic evolution, speciation, diversification, and adaptation (Glasauer and Neuhauss, 2014; Macqueen and Johnston, 2014; Warren *et al.*, 2014), as it negatively affects genomic stability, polyploids are most likely to return to a diploid state via a process termed rediploidization (Veitia and Bottani, 2009; Van de Peer, Mizrachi and Marchal, 2017).

However, in Atlantic salmon, rediploidization is yet to be complete, with large swathes of the genome still in high homology with their duplicated region (Robertson *et al.*, 2017) which can complicate genomic analysis and selection for various reasons. These include (1) uncertainty in the *S.salar* reference genome, particularly in regions with incomplete rediploidization, (2)

conversely, reduced confidence in mapped reads, and (3) difficulty in distinguishing (possibly divergent) gene expression signals between (possibly multiple) gene copies. Consequentially, most pipelines aim to remove and filter out highly homogenous regions and duplicates to facilitate analysis which has itself led to a general understudying of such regions (Houston and Macqueen, 2019) .

1.2.3. Husbandry advancements and recirculating aquaculture systems

The increases in industry productivity has also been enabled, in part, by husbandry and technological advancements which increased control over the rearing environment. The provision of constant light and temperature, coupled with an artificial winter photoperiod, has allowed a decrease in the length of FW development by boosting growth rates, as well as producing smolts continually throughout the year. The combined effect means smolting is now often achieved in under-yearling parr (Thrush, Duncan and Bromage, 1994; Björnsson *et al.*, 2000; Ellis *et al.*, 2016). Furthermore, while the rearing environment during SW is primarily sea-cages, the FW farming environment is comparatively more variable (Ellis *et al.*, 2016). These range from floating net-pens in FW lochs, to land-based flow-through (tanks, troughs, and raceways) and recirculating systems (recirculating aquaculture systems or RAS). Though there is geographical variation in their use, there has been progressive movement towards land-based systems over recent years (Bergheim *et al.*, 2009).

Wider implementation of RAS for the grow-out phase of some or all species is now suggested to continue the increase in aquaculture production, fulfilling the growing demand for sea food globally not met by capture fisheries while minimising the industry's environmental impact (Ebeling and Timmons, 2012; Clarke and Bostock, 2017; FAO, 2020). Advantages of RAS include its expandability, flexible location, safety, quality, and consistency of fish produced, biosecurity, and environmental stability (Ebeling and Timmons, 2012; Kolarevic *et al.*, 2014; Mota *et al.*, 2019). Furthermore, as a closed system with water pumped in and recycled (Figure 1-4), RAS reduces land and water use while mitigating impacts of escapees, disease, and waste (Thorarensen and Farrell, 2011; Ebeling and Timmons, 2012).

This process, however, requires physical, chemical, and biological filtrations to maintain water quality, with the main targets including suspended solids (SS), carbon dioxide (CO₂), and ammonia (NH₃) (Ebeling and Timmons, 2012) (Figure 1-4). If not monitored and controlled, accumulation of such substances can affect fish physiology and welfare. Large economic investments are therefore necessary for both the set up and running of such systems (Liu *et al.*, 2016), meaning the feasibility of their use can be limited. To counteract this, fish are reared at higher densities, provided the system can adequately process the increased fish derived waste (Thorarensen and Farrell, 2011).

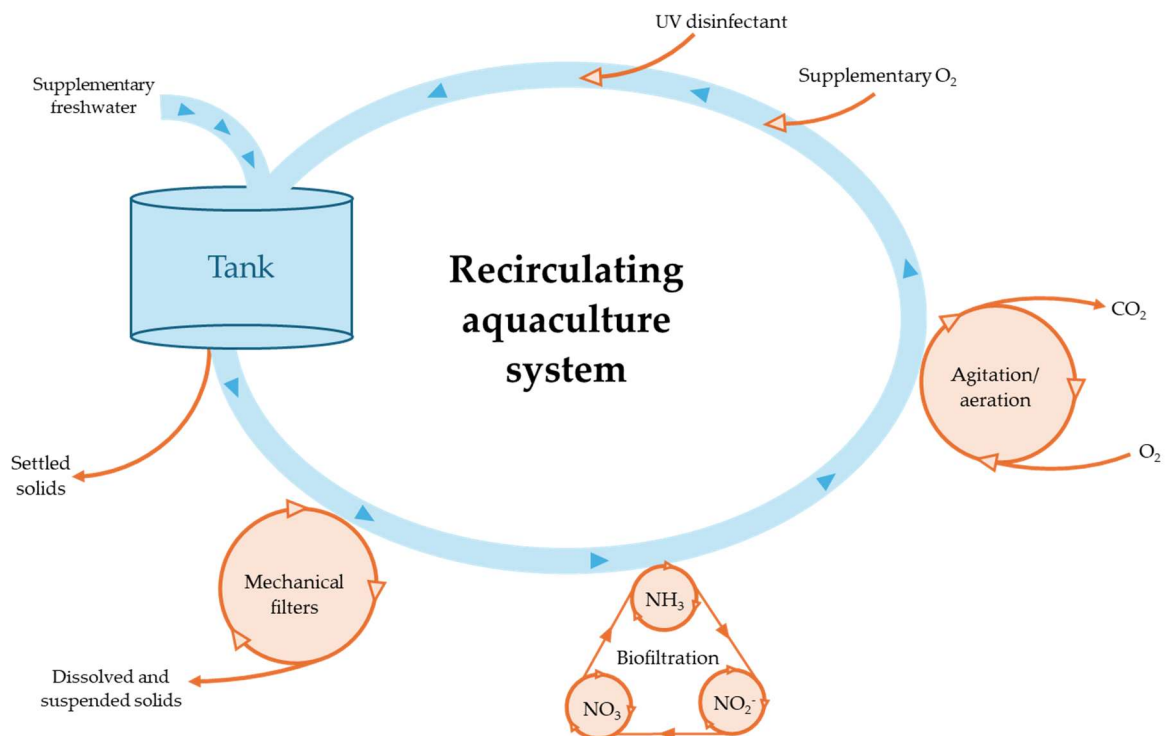


Figure 1-4 Illustration of a recirculating aquaculture system. Orange sections indicate steps to maintain water quality.

1.2.3.1. Water quality in RAS

Suspended solids

Suspended solids (SS) in aquaculture systems can originate externally or internally, be inorganic or organic and vary in chemical composition, shape, and size. These parameters relate to the system employed. In closed systems most SS are internally produced organic matter originating from uneaten food or faecal matter (Schuman and Brinker, 2020). The characteristics of SS relate to feed composition, with their particle size distribution also affected by the level of water velocity (Gorle *et al.*, 2018; Schumann and Brinker, 2020). In

recirculating systems, large-settled particles are removed from tanks, and remaining SS are filtered out. Dissolved and fine SS are, however, particularly hard to remove and can potentially accumulate with water reuse (Ebeling and Timmons, 2012; Becke *et al.*, 2019; Schumann and Brinker, 2020). Overall, in RAS there is a predominance for fine internally produced organic SS. Though a wide range of particle loads have been reported, there is a tendency for this to be higher in RAS compared to flow-through, semi-RAS and open water net-pens (Schumann and Brinker, 2020).

The direct effect of SS is physical damage, especially to delicate structures such as the gills. Indirect effects include changes to water clarity affecting feeding behaviour, and growth of heterotrophic bacteria (Becke *et al.*, 2017, 2018). Bacterial load is linearly related to the particle load of water, providing a substrate and food source for bacterial growth which can increase infection pressure, stress, formation of fatal substances (e.g., hydrogen sulphide) as well as altering the flavour of fish produce (Davidson *et al.*, 2014; Gorle *et al.*, 2018). Experimentally, however, reports have found the effects of SS on Rainbow trout, *Oncorhynchus mykiss*, in RAS to be minimal in the short term (Becke *et al.*, 2017) and chronic (Becke *et al.*, 2018) when relating to bacterial load, physical damage, and haematological parameters.

Ammonia, nitrites and nitrates

SS can also increase the concentration of nitrites (NO_2^-) and ammonia (ionized NH_4 or toxic unionized NH_3) via nutrient leaching or decreasing the efficiency of the biofilters used for dissolved nitrogen's removal. Specifically, fine SS can both clog these filters, or facilitate the growth of heterotrophic bacteria within, outcompeting nitrifying microorganisms (Kocour Kroupová *et al.*, 2018; Schumann and Brinker, 2020). Biological filters aim to oxidise ammonia and nitrites to nitrate (NO_3^-) as they are toxic. These toxic substances would otherwise oxidise haemoglobin to methaemoglobin, reducing the oxygen carrying capacity of the blood and potentially causing high mortality (Kocour Kroupová *et al.*, 2018; Schumann and Brinker, 2020). Ammonia has a range of other physiological implications too (see Jensen (2003) or Kocour Kroupová *et al.* (2018)).

This reaction also consumes water alkalinity, which requires continual replacement, usually by adding sodium bicarbonate (NaHCO_3), to maintain biofilter efficiency (Kolarevic *et al.*,

2014). As ammonia and chloride uptake are related, species, like salmonids, with high chloride absorption are more sensitive to ammonia concentration (Kocour Kroupová *et al.*, 2018). Though the critical concentration of nitrite is widely accepted as $\sim 0.0125 \text{ mgL}^{-1}$, Rainbow trout (*O. mykiss*) reared in RAS have shown no detrimental effects when exposed to much higher concentrations (Becke *et al.*, 2019), with similar results reported in post-smolt Atlantic salmon (Davidson *et al.*, 2017).

Oxygen, carbon dioxide, and pH

Oxygen is the primary limited resource in RAS but this can be overcome by supplying pure oxygen to systems after which the concentration carbon dioxide (CO_2) can begin to negatively impact growth and performance (Ebeling and Timmons, 2012). CO_2 can accumulate as a respiratory product of fish and nitrifying bacteria in biofilters (Ebeling and Timmons, 2012; Fivelstad, 2013; Summerfelt *et al.*, 2014). Acute exposure to high CO_2 concentrations increases plasma CO_2 partial pressure, lowers pH, and reduces oxygen transportation. Additionally, it can cause hyperventilation, increased adrenaline levels and reduced plasma chloride, among other effects. Chronic exposure to high concentrations reduces growth, feed conversion efficiency, feed intake and condition factor as well as development of nephrocalcinosis and thinner mucus and dermal tissues upon SW transfer (Fivelstad, 2013; Summerfelt *et al.*, 2014; Mota *et al.*, 2019). However, the occurrence of these effects is not consistent, as seen in the incidence of nephrocalcinosis in salmonids (Fivelstad, 2013; Good *et al.*, 2018; Mota *et al.*, 2019). More recently, growth rate was shown as linearly negatively related to CO_2 concentration (Fivelstad, 2013; Mota *et al.*, 2019, 2020), with negative effects felt below current safety thresholds ($\text{CO}_2 < 20 \text{ mgL}^{-1}$) (Mota *et al.*, 2020). Furthermore, the effects of FW CO_2 were seen after SW transfer, indicating SW performance relies, in part, on previous culture environmental conditions (Mota *et al.*, 2019).

As mentioned, CO_2 is part of the carbonate buffering system in blood but also acts in the same capacity for water's carbonate buffering systems. Consequentially, CO_2 concentration of water is affected by and affects other water characteristics like pH, alkalinity, O_2 , and hardness (Fivelstad, 2013). For example, pH decreases with CO_2 concentration due to formation of carbonic acid. This is itself detrimental to fish, but can also cause dissolved metals, such as aluminium, copper and zinc, to increase in their toxicity (Fivelstad, 2013; Summerfelt *et al.*,

2014). While CO₂ is removed through agitation of water and increased exposure to air where it comes out as a gas due to differences in partial pressure between mediums, for intensive farms with high stocking density this may not be sufficient to maintain safe levels (Summerfelt *et al.*, 2014). Instead, the addition of alkalis (e.g., bicarbonate) can buffer against decreased pH (Kolarevic *et al.*, 2014), with smolts in high alkali FW seen to be more tolerant to high concentrations of dissolved CO₂ (Fivelstad, 2013)

1.3.Genotype-environment interactions

Given the aquaculture industry structure, offspring from the same breeding population are often widely disseminated and reared across a range of production environments, varying in terms of technology, husbandry regimes and geographic location. For Atlantic salmon, this environmental variation is compounded by the varied rearing environment employed throughout the production cycle. Such a model can prove difficult where genotype-environment interactions (GxE) are present, defined as different sensitivities to the same macroenvironmental change between genotypes (Falconer, 1952; Falconer and Mackay, 1996). Two components of GxE have been described, scaling effect and re-ranking (Falconer and Mackay, 1996; Sae-Lim *et al.*, 2016) (Figure 1-5). The former is a measurement of heterogeneous genetic variance between environments, while the latter indicates that there is no single best performing genotype across environments (Falconer, 1952; Sae-Lim *et al.*, 2016; Agha *et al.*, 2018) (Figure 1-5). While the scaling effect can be more readily accounted for in quantitative models or regulated by husbandry, re-ranking is a serious concern in an aquaculture setting (Campos-Montes *et al.*, 2009; Bangera *et al.*, 2015; Sae-Lim *et al.*, 2016).

GxE has been typically identified by examining variations in genetic parameters across environments, including heritability, genetic variance, and genetic correlations amongst traits within each environment (Sae-Lim *et al.*, 2014). However, a key parameter is the genetic correlation between the same trait but in different environments (r_{gxe}) as it is widely used to quantify re-ranking. While ideally this value would approximate one indicating the performance of genotypes is the same across environments (e.g. Figure 1-5, panel A), values less than this suggest selective gains will not be realised across farms (Sae-Lim *et al.*, 2016). Specifically values less than 0.8 were suggested by Robertson (1959) as threshold for GxE being of biological significance to a program of selection. Others have suggested boundaries for

GxE, such as Navarro *et al.* (2009b) who described 0-0.40 as low, 0.45-0.55 as medium and 0.60-1 as high genetic correlations; and Ponzoni *et al.* (2008) described genetic correlations of 0.5, 0.7 and 0.9 as severe, moderate, and insignificant GxE, respectively.

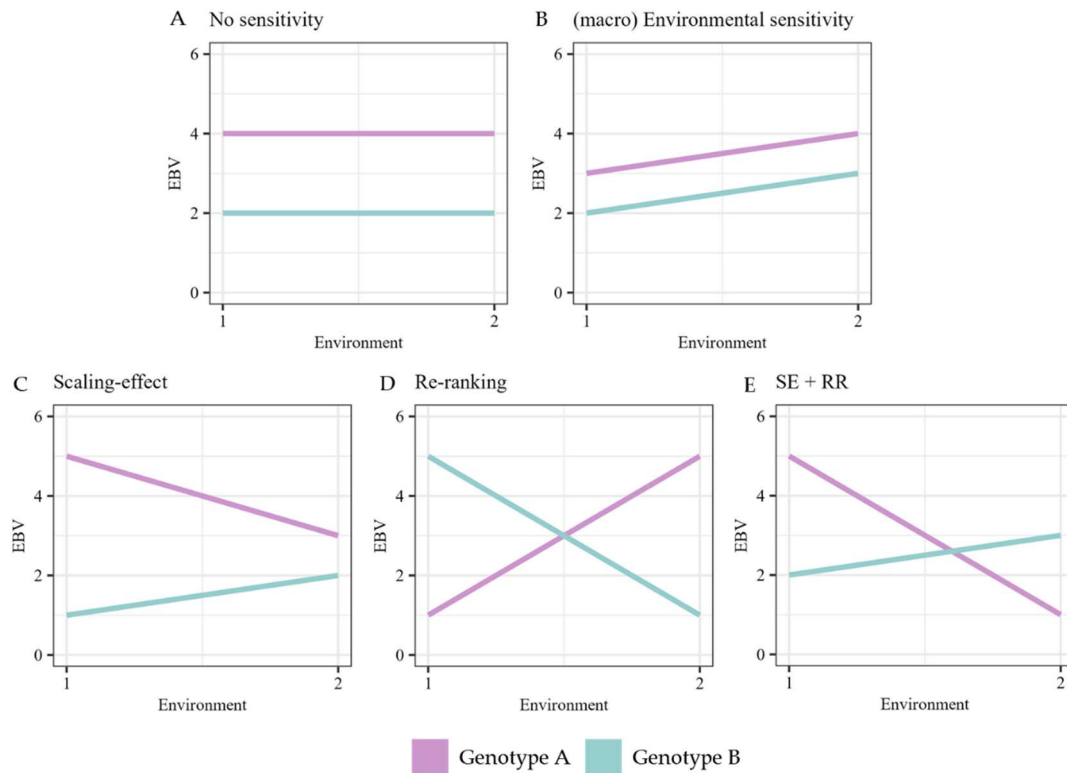


Figure 1-5 Visualization of different components of genotype-environment interactions as the estimated breeding value (EBV) of two different genotypes between two environments. SE is scaling effect and RR ranking. Re-drawn from Sae-Lim *et al.*, 2016.

More recently, a new benchmark was suggested by Sae-Lim (2013) as a correlation below 0.7. This threshold relates to the ‘break-even correlation’ which describes the intersection of predicted genetic gains under two different breeding programs. As such, where values fall below this threshold it would be theoretically better to employ two smaller environment-specific breeding programs as opposed to a single larger general program (Mulder *et al.*, 2006; Sae-Lim, 2013; Sae-Lim *et al.*, 2016). However, formation of new breeding programs would be costly and other options to account for GxE involve husbandry manipulation to reduce environmental differences between environments, introduction of index selection considering performance across environments, or establishment of localised multiplier or sib-testing stations (Mulder *et al.*, 2006; Sae-lim *et al.*, 2015; Omasaki *et al.*, 2016; Agha *et al.*, 2018; Van Sang *et al.*, 2020). Final breeding decisions will reflect the trade-off between costs associated with these actions and benefits to profitability and productivity of farms, something which

will be specific to the species, local industry, and farm purpose (Sae-Lim, 2013; Sae-Lim *et al.*, 2016).

1.3.1. Statistical approaches for GxE

At its simplest GxE can be studied through modelling an interaction between the genetic and environment terms (McKay, Friars and Ihssen, 1984; Wild *et al.*, 1994). However, this provides little information on the nature of the relationship. Instead, GxE are commonly identified through use of statistical models which provide estimates of genetic (co)variance structures. As these include genetic variance and co-variance, assessment of GxE can be made by quantification of genetic correlation (r_{gxe}). These models include (1) bi- or multi- variant models, (2) reaction norms and (3) factor analytic structures (reviewed by Sae-Lim *et al.* (2016)).

In use of bi- and multi-variant models, the same trait measured in different environments is treated as two independent traits allowing estimation of genetic correlation between them (Falconer, 1952; Kolmodin, 2003). Alternatively, reaction norms model phenotypic performance as a function of the environment, typically expressed as a polynomial. The coefficient of the polynomial function is assumed to be under genetic influence (Kolmodin, 2003; Kolmodin *et al.*, 2003). Most studies model the relationship between phenotype and environment as linear, or in other words a first-order polynomial function (though second- and third- order relationships have been observed) (Kolmodin, 2003; Kolmodin *et al.*, 2003; Rauw and Gomez-Raya, 2015). In this context, GxE can be understood as genotypes' regression slopes crossing along an environmental gradient (e.g. Figure 1-5 panels D and E). Modelling of the genetic (co)variance of this polynomial coefficient then allows the quantification of r_{gxe} (Sae-Lim *et al.*, 2016).

More recently, models have made use of factor analytic (FA) structures (Sae-Lim *et al.*, 2016). This approach identifies two variables which underpin genetic (co)variation: (1) specific effects and (2) latent common factors (LCF), as well as their additive genetic components. Specifically, their additive genetic (co)variance structure consists of a matrix of factor loadings and diagonal matrix of specific variances (variance left unexplained by LCF). The squared factor loadings indicate the amount of additive genetic variance under a specific environment

which is explained by the LCF. High factor loadings indicate that the LCF explains much of the additive genetic variance of an environment (Meyer, 2009; Sae-Lim *et al.*, 2014). GxE is identified as different measurements of additive genetic variance across environments (Meyer, 2009; Sae-Lim *et al.*, 2014, 2016).

Importantly, though both re-ranking and scaling effect can be distinguished in use of bi- or multi- variant models, as the environmental effect included in models is the holistic effect of many possibly interacting variables, it is not possible to identify potential causal environmental factors (Sae-Lim *et al.*, 2016). In contrast, FA and reaction norm approaches can allow identification of possible causal environmental variables driving GxE between environments (Sae-Lim *et al.*, 2016). For FA approaches the strength of correlations between LCF loadings and different environmental variables can indicate likely variables. Whereas for reaction norms this can be done via the comparison of the loglikelihood of models with different environmental variables fit (Sae-Lim *et al.*, 2014).

1.3.2. Trait uniformity and micro environmental sensitivity

More recently, research has focused on microenvironmental sensitivity, defined as an individual's ability to buffer against unknown biotic and abiotic disturbances within a single macroenvironment, as well as developmental or endogenous disturbances (Sonesson, Ødegård and Rønnegård, 2013). Approaches to study micro environmental sensitivity often quantify it as the environmental variance remaining after all known fixed and random effects are accounted for (i.e. V_e). Practically, this is often assumed to be the residual variance (i.e. V_r) estimated from quantitative analysis (i.e. Eq 1-2) of a given trait. In methodological approaches this often represents the first of two models used, which analyses the trait of interest (mean model). This is followed by a second (variance model) where the estimated residual variance (V_e or V_r) obtained from the mean model becomes the trait of interest, which is used to identify the additive genetic components of residual variance (A_v) (Hill and Mulder, 2010; Sae-Lim *et al.*, 2016; Iung *et al.*, 2020). When this is heterogeneous, known as genetic heterogeneity of residual variance, microenvironmental sensitivity is considered to be present (Mulder *et al.*, 2009; Hill and Mulder, 2010).

Importantly, when A_v is present, there is potential to reduced trait variance via selection for individuals with low sensitivity (Agha *et al.*, 2018). Key parameters include heritability of variance (h_v^2) defined as the accuracy of predicted EBV of the residual variance, and coefficient of genetic variation (CGV_v) or standard deviation (CGV_{sd}) representing how much the residual variance or standard deviation changes when changing the residual variance or standard deviation by one genetic unit (Iung *et al.*, 2020).

Increasing trait uniformity is related to ideas of robustness, which is becoming an increasingly relevant trait in aquaculture breeding programs (Knap, 2008; Janhunnen *et al.*, 2012; Berghof, Poppe and Mulder, 2019), but carries several other practical advantages in of itself. Taking body weight as an example, increased uniformity can (1) boost market value via customer preference, especially when the species is sold commercially whole, (2) reduce handling and processing as well as the need for common husbandry practices such as grading, all reducing production costs and (3) improve welfare via reduced likelihood or strength of social hierarchies, which can further exacerbate size variation (Khaw *et al.*, 2016; Marjanovic *et al.*, 2016; García-Ballesteros *et al.*, 2021; Garcia *et al.*, 2021; Vu *et al.*, 2021).

From the limited studies on microenvironmental sensitivity in aquaculture (Table 1-1), only small, though significant, estimates of additive genetic components of residual variance have been reported (Sae-Lim *et al.*, 2016; Iung *et al.*, 2020). It should be considered that some of this observed within-family heterogeneity of variance may simply have been a result of the scaling effect where variance increases as a function of the trait mean (Sonesson, Ødegård and Rönnegård, 2013). To overcome this, some studies have transformed phenotypic data to break the dependency between a trait and its variance. Methods include log-, square-root-, or box-cox transformations, with results often differing between transformed and untransformed data (Khaw *et al.*, 2012; Sae-Lim, Kauser, *et al.*, 2015; Vu *et al.*, 2021) (Table 1-1). Indications of significant additive variance component of residual variance after such transformations have highlighted the presence of within-family heterogeneity beyond scaling effect (Sonesson, Ødegård and Rönnegård, 2013).

The practical impact of variance heterogeneity has been demonstrated by Sonesson, Ødegård and Rönnegård (2013), who showed that even with low estimates of additive genetic variance of residual variance (0.03-0.04), the variance of families with extremely high trait value would

be approximately three times greater than that of the families with extremely low trait value. Similarly, while evidence of selecting for uniformity is scant in aquaculture, results from lines selected for high and low variance suggests its plausibility (Iung *et al.*, 2020). It should be noted that selection for a trait's uniformity in isolation from the trait itself would be a difficult task. Therefore, index selection, which can consider both the (transformed or untransformed) trait as well as its uniformity, is often suggested as a viable option (Khaw *et al.*, 2012; Vu *et al.*, 2021).

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Table 1-1 Review of microenvironmental studies in fish. All values have been rounded to 2 decimal places for consistency. V_e : Environmental (residual) variance; A_v : additive genetic variance of V_e ; h_m^2 : narrow sense heritability of the trait; h_v^2 : narrow sense heritability of trait variance; r_{vm} : genetic correlation between the trait mean and its variance; CGV_v : genetic coefficient of variance for the residual variance; QGM: quantitative genetic model; REML: restricted maximum likelihood; Ln: natural logarithm; $BW_{2\text{ years}}$: body weight at 2 years; BW_H : body weight at harvest; BW_T : body weight at tagging; WW_H : wet weight at harvest; DHGLM: double hierarchical general linear model; A matrix: numerator relationship matrix (pedigree); GRM: genomic relationship matrix (e.g. SNP data); GIFT: genetically improved farmed tilapia.

Citation	Species	Location	Environment	Trait	Variance Model Specification				Parameter Estimates				
					QGM for V_e	V_e	Method	Statistical model of A_v	h_m^2	A_v	h_v^2	r_{vm}	CGV_v
Janhunen et al., 2012	<i>Oncorhynchus mykiss</i>	Finland	Indoor tanks	$BW_{2\text{ year}}$	Exponential	$\text{Ln}(\text{residuals}^2)$	2-stage REML ^M	Animal	0.35 (0.05)	1.81e ^{8C}	0.02 (6.0x10 ⁻³) ^{CL}	-1.57 (0.04)	0.38 ^C
Sonesson et al., 2013	<i>Salmo salar</i> (AquaGen)			BW_H	Exponential	Squared residuals ^A	DHGLM	4*sire-dam	0.36 (0.04)	0.17 (0.03)		0.42 ^J	
				$\log BW_H$					0.32 (0.04)	0.14 (0.04)		-0.17 ^J	
				$\sqrt{BW_H}$					0.35 (0.04)	0.13 (0.03)		0.14 ^J	
Agha et al., 2015	<i>Oreochromis niloticus</i> (GIFT)	Malaysia	Pond	$\sqrt{BW_H}$	Exponential	$\text{Ln}(\text{residuals}^2)$	Bayesian ^I	Animal	0.29 (0.01)	0.45 (0.01)		-0.53 (0.06)	
		India							0.18 (0.01)	0.70 (0.23)		-0.03 (0.14)	
		China							0.31 (0.01)	1.90 (0.28)		-0.07 (0.01)	
Khaw et al., 2015	<i>Oreochromis niloticus</i> (GIFT)	Malaysia	net-cages in earthen ponds	BW_H (+box-cox)	Additive	Standard deviation	2-stage REML ^M	4*sire-dam	0.59 (0.09) ^H	6.9x10 ⁻³ (2.2x10 ⁻³)	0.23 (0.07) ^H	0.10 (0.18)	0.17 ^G
Sae-Lim et al., 2015	<i>Oncorhynchus mykiss</i>	Finland	Inland FW	BW_H (+ standardised ^E)	Exponential	Linearized variance ^B	DHGLM	4*sire-dam	0.26 (0.03)	6.8x10 ^{-3C}	0.01 ^C	0.30 (0.15)	0.22 ^C
			Commerical sea-cages, Blatic sea,	$\log BW_H$					0.17 (0.02)	4.0x10 ^{-4C}	0.02 ^C	0.79 (0.13)	0.30 ^C
				BW_H (+ standardised ^E)					0.22 (0.03)	8.5x10 ^{-3C}	0.01 ^C	-0.83 (0.10)	0.19 ^C
				$\log BW_H$					0.12 (0.02)	3.0x10 ^{-4C}	0.01 ^C	-0.62 (0.21)	0.17 ^C
Marjanovic et al., 2016	<i>Oreochromis niloticus</i> (GIFT)	Malaysia	net-cages in earthen ponds	BW_H	Exponential	Linearized variance ^B	DHGLM	4*sire-dam	0.25 (0.04)	0.34 (0.07)		0.6 (0.09)	0.58
				length					0.25 (0.5)	0.16 (0.04)		0.11 (0.16)	0.39
				depth					0.3 (0.5)	0.18 (0.04)		0.37 (0.13)	0.42
				width					0.3 (0.5)	0.20 (0.05)		0.20 (0.15)	0.45
Sae-Lim et al., 2017	<i>Salmo salar</i> (Nofima AS & SalmoBreed)	Norway	Experimental tanks with sea lice challenge test at 16 months	BW_H (+ standardised ^E)	Exponential	Linearized variance ^B	DHGLM-A matrix	4*sire-dam	0.27 (0.10)	0.06 ^C	0.04 (0.02) ^{CL}	0.95	0.48 (0.11) ^L
				$\log BW_H$					0.33 (0.10)	5.0x10 ^{-4C}	0.02 (0.01) ^{CL}	-0.09	0.30 (0.10) ^L
				BW (+ standardised ^E)		Linearized variance ^B	DHGLM - GRM	4*sire-dam	0.30 (0.10)	0.06 ^C	0.04 (0.02) ^{CL}	0.95	0.52 (0.12) ^L
				$\log BW_H$					346 (0.11)	5.0x10 ^{-4C}	0.01 (0.01) ^{CL}	0.02	0.30 (0.10) ^L
Sae-Lim et al., 2020	<i>Cyclopterus lumpus</i>	Norway	Circular tanks	BW_T (+ standardised ^E)	Exponential	Linearized variance ^B	DHGLM	4*sire-dam	0.57 (0.10)	0.03 ^C	0.01(9.0x10 ⁻³) ^C	-0.06 (0.25)	0.46
				$\log BW_T$					0.61 (0.15)	1.0x10 ^{-3C}	0.02 (0.02) ^C	-0.81 (0.11)	0.64

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García-Ballesteros <i>et al.</i> , 2021	Litopenaeus vannamei (CAMANICA S.A)	Nicaragua	Separate family rearing/breeding environment	BW _H	Exponential	Squared residuals ^A	DHGLM (univariate analysis for each environment)	4*sire-dam	0.38	0.06 (0.01)	0.02 ^C	0.11 (0.06)	0.25 (0.01)
			Geographically varied ponds	BW _H		0.39	0.06 (0.01)	0.02 ^C	0.07 (0.07)	0.23 (0.02)			
			Separate family rearing/breeding environment	BW _H		Squared residuals ^A	DHGLM (Multivariate analysis across environments)	4*sire-dam	0.38	0.06 (0.01)	0.02 ^C	0.11 (0.06)	0.25 (0.01)
			Geographically varied ponds:	BW _H		0.39	0.06 (0.01)	0.02 ^C	0.05 (0.07)	0.24 (0.01)			
Garcia <i>et al.</i> , 2021	Litopenaeus vannamei	Mexico	Low density earthen ponds	BW _H	Exponential	Linearized variance ^B	DHGLM	4*sire-dam	0.15 (0.02)	0.11(0.02)	0.03 (0.01) ^C	0.17 (0.13)	0.35 (0.03) ^C
			High density concrete recirculation systems	BW _H					0.24 (0.02)	0.03 (0.02)	0.01 (3.0x10 ⁻³) ^C	0.34 (0.22)	0.17 (0.05) ^C
			Combined	BW _H					0.07 (0.01)	0.08 (0.02)	0.02 (3.0x10 ⁻³) ^C	-0.23 (0.08)	0.28 (0.02) ^C
Vu <i>et al.</i> , 2021	Crassostrea angulata	Vietnam	Co-culture environment	WW _H (+ standardised ^D)	Exponential	Linearized variance ^B	DHGLM ^F	Animal	0.15 (0.01)	0.19	0.07 (0.01)	~ 1 in both environment s. – 1 after log transformation.	
				logWW _H					0.31 (0.01)	0.02	0.01 (5.0x10 ⁻⁴)		
			oyster cultivation only	WW _H (+ standardised ^D)					0.08 (0.01)	0.14	0.06 (0.01)		
				logWW _H					0.25 (0.01)	0.05	0.02 (0.01)		
Campos <i>et al.</i> , 2024	Oreochromis niloticus	Brazil	Net cages	BW _H	Additive	Ln(residuals) ³	2-stage REML ^M	Animal	0.31 (0.06)	6.15e ⁶ (2.21e ⁶)	0.02 (9.0x10 ⁻³)	-0.43 (0.15)	0.29 (0.07)

^A as in Rönnegård et al. (2010)

^B as in Felleki et al. (2012)

^C converted to additive scale

^D standardized by division of 10

^E standardized as $\mu \sim (0,1)$

^F factor analytic structure included to calculate genetic correlation

^G CGV_{SD}

^H measured at the level of familygroup

^I HPD rather than s.e

^J pearson rather than genetic correlation

^K if not explicitly stated in the reference, then based on equations layed out in Iung et al. (2020)

^L s.e based off equations in Mulder, Bijma and Hill (2007)

1.4.Epigenetics

When first introduced by Waddington (1942), epigenetics considered how phenotypes arise through development, though this definition has been expanded and modified several times, contemporarily it is widely reported as chromosome-bound, mitotically or meiotically heritable changes to gene expression, independent of DNA changes itself (Deans and Maggert, 2015; Best *et al.*, 2018; Eirin-Lopez and Putnam, 2019).

1.4.1.Epigenetic mechanisms

Various epigenetic mechanisms have been characterised. Firstly, there is chromatin remodelling which can alter gene expression by causing shifts between transcriptionally silent heterochromatin and transcriptionally open euchromatin. This can be driven by either (1) small covalent modification to histone side chains, or (2) ATP-driven nucleosome-remodelling enzyme complexes (Clark and Rager, 2020). In the former, addition of small modifications can alter histone-DNA binding, changing nucleosome packing and chromatin state. While a wide variety of modifications have been identified (see Kouzarides, 2007; Sadakierska-Chudy and Filip 2015 or Lawrence, Daujat and Schneider, 2016), predominantly understood and studied is the enzymically catalysed acetylation/deacetylation and methylation/demethylation of lysine and arginine residuals on the N-terminal tails of histone proteins H3 and 4 (Rice and Allis, 2001). Alternatively, these modifications can block or bind to proteins capable of further modifying nucleosomes (Rice and Allis, 2001; Kouzarides, 2007; Labbé, Robles and Herraiz, 2017). In the latter, these complexes affect nucleosome positioning in DNA via recruitment by sequence-specific transcription factors, acting as co-activators or co-repressors of transcription (Clark and Rager, 2020).

Secondly, there are the transcripts of non-coding RNAs (ncRNAs), whose regulatory role is only now being recognised, which impact gene expression post-transcriptionally by acting on the abundance of RNA transcripts (Clark and Rager, 2020). ncRNAs are diverse and can be initially categorised as long (lncRNA, >200 nucleotides) or small (small ncRNA, <30 nucleotides) (Clark and Rager, 2020). Small ncRNAs can be further classed as microRNA (miRNA), Piwi-interacting RNA (PiRNA) or small interfering RNA (siRNA). Small ncRNAs and can alter gene expression as a part of the RNAi pathway where, in brief, they direct the

action of RNA-induced silencing complex (RISC) or PIWI proteins to complementary sequences and repress translation, drive mRNA degradation, or alter chromatin state (Clark and Rager, 2020). Long ncRNAs are a highly variable group and can be classed based on their proximity to protein coding genes and genomic location, cellular/subcellular localization, or archetype much of which affects their regulatory behaviour (Ponting, Oliver and Reik, 2009; Kaikkonen, Lam and Glass, 2011; Sriyothi *et al.*, 2018; Clark and Rager, 2020). However, they are generally seen to affect this post-transcriptionally as well as interacting with DNA and protein molecules (Clark and Rager, 2020). In addition, specific ncRNAs associated with promoter and enhancer regions have been characterised (Kaikkonen, Lam and Glass, 2011; Eirin-Lopez and Putnam, 2019).

1.4.1.1.DNA methylation and gene expression

In addition to these other epigenetic mechanisms there is DNA methylation (DNAm), which is by far the most studied and understood. Though not the only form of genomic methylation, DNAm is widely characterised as 5-methylcytosine (5mc), where methyl groups, provided by donor S-adenosylmethionine (SAM), are added to C5 position of cytosine (Figure 1-6). This is an active process catalysed by DNA methyltransferases (DNMTs), which include DNMT1 for maintenance and DNMT3a and b for *de novo* methylation (Gavery and Roberts, 2017; Best *et al.*, 2018). Specifically, DNMT 3 a and b are concerned with the methylation of cytosines which have never been methylated before, such as newly synthesised DNA strands or methylation during early embryonic development. In contrast, DNMT1 acts to maintain methylation through the cell cycle and DNA replication, ensuring methylation of the parent strand of DNA is preserved (Clark and Rager, 2020).

Removal of methyl groups is also possible and can occur passively or actively (Figure 1-6). Passive demethylation occurs when methylation patterns are eroded by successive cellular divisions in the absence of its maintenance by DNMT1 (Best *et al.*, 2018; Clark and Rager, 2020). On the other hand, in active demethylation the removal of methyl groups is enzymatically catalysed (Figure 1-6). Two pathways have been proposed for this catalytic removal of methyl groups. In the first, 5mc is oxidised by enzymes from Ten-Eleven Translocation (TET) methyl-cytosine dioxygenase family, generating a series of intermediates (5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC))

which are removed by thymine DNA glycosylase (TDG) and thymine-mediated excision and base extension repair (BER). In the second pathway, the deamination of 5mc occurs by activation-induced cytosine deaminase (AICDA) and apolipoprotein B mRNA editing enzyme catalytic polypeptide (APOBEC), followed by BER (Figure 1-6). Consensus on this latter pathway is yet to be fully resolved (Best *et al.*, 2018).

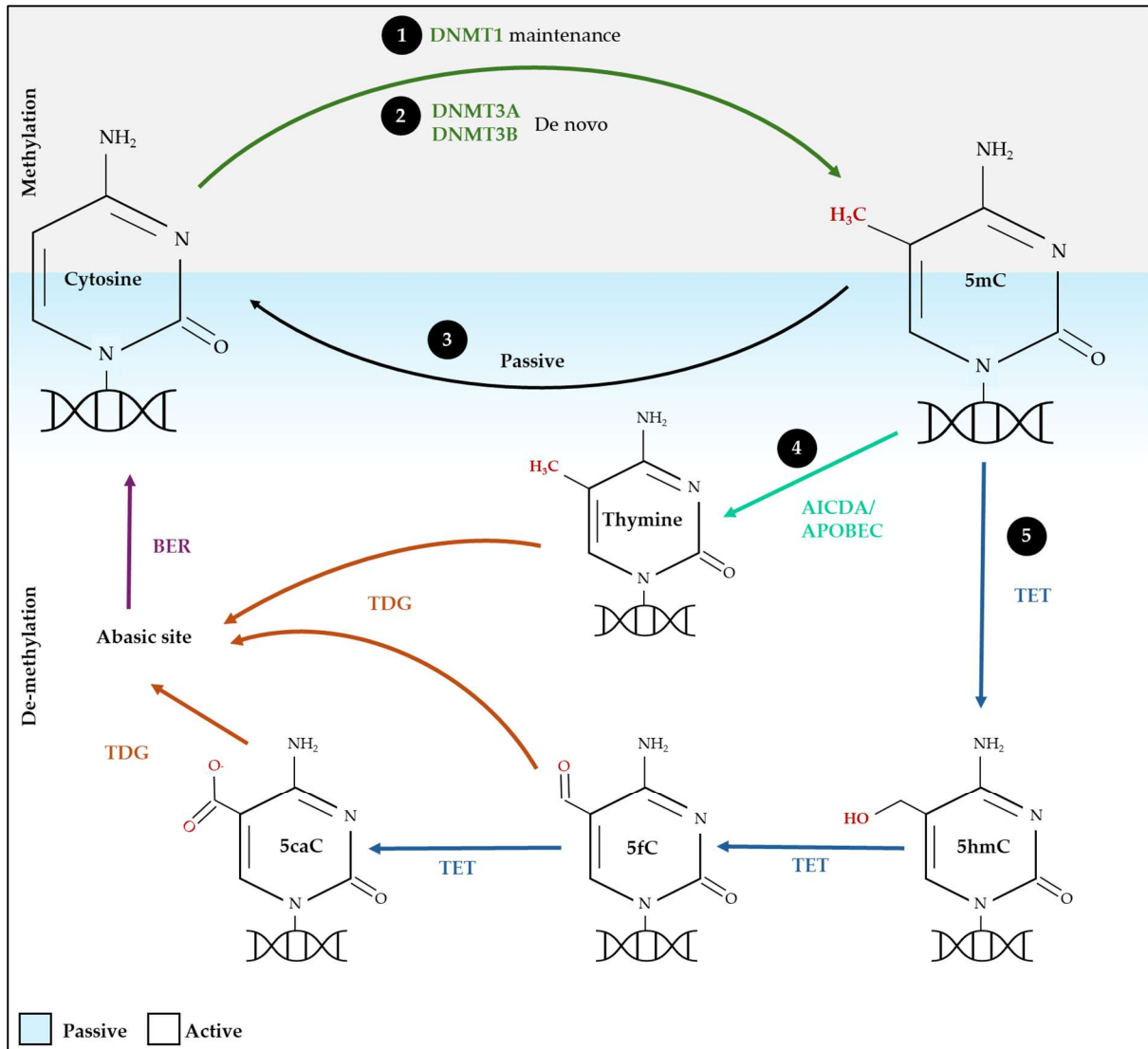


Figure 1-6 Pathways for methylation (1 and 2) as well as passive (3) and active (4 and 5) demethylation. Arrows show direction of action and coloured to corresponding enzymes. In this DNMT is de-methyl transferase, TET Ten-Eleven Translocation methyl-cytosine dioxygenase family, AICDA is activation-induced cytosine deaminase, APOBEC is apolipoprotein B mRNA editing enzyme catalytic polypeptide, TDG is thymine DNA glycosylase and BER is base extension repair. 5mc is 5-methyl-cytosine, 5hmC is 5-hydroxymethylcytosine, 5fC is 5-formylcytosine and 5caC is 5-carboxylcytosine. Based on information presented in Gong and Zhu, (2011); Best *et al.*, (2018); Liu *et al.*, (2021).

While the majority of the vertebrate genome is unmethylated, methylated regions are non-uniformly distributed throughout and are instead predominantly found at 5'-cytosine-phosphate-guanine-3' (CpG) sequences (Eirin-Lopez and Putnam, 2019). However, while the

majority of CpG sites are methylated, only a minority of methylated CpG are found in CpG islands (CpGi), which are regions characterized with high density of CpG sites. Instead CpGi are somewhat protected from methylation (Long *et al.*, 2016; Ambrosi, Manzo and Baubec, 2017; Clark and Rager, 2020). This pattern is in part a result of the mutagenic effect of DNAm, which through demethylation can lead to cytosine's transition to a thymine base. On an evolutionary timescale, GC content, and consequently DNAm, has been progressively lost (Elango and Yi, 2008; Ambrosi, Manzo and Baubec, 2017). It should be noted that the vast majority of DNAm research in vertebrates has been performed on model mammalian species, and how these differ in other vertebrates, such as fish, has not been resolved and may have implications for its experimental study (Wellband *et al.*, 2021).

The importance of genomic methylation patterns, however, lies in its ability to regulate gene expression through: (1) methyl group interactions with the major groove of the DNA helix enhancing or blocking DNA binding proteins and transcriptional machinery, or (2) alterations to methyl-CpG-binding domain proteins and their recruitment of further proteins with transcriptional effects (Feil and Fraga, 2012; Eirin-Lopez and Putnam, 2019). While the traditional view of DNAm is to repress gene expression, emerging evidence suggests that its effect is largely context dependent (Brenet *et al.*, 2011; Ambrosi, Manzo and Baubec, 2017; Anastasiadi, Esteve-Codina and Piferrer, 2018; Mukiibi *et al.*, 2022).

1.4.2. The environment, DNAm, and phenotypic expression

As described, the epigenome can alter patterns of gene expression independent of changes to the DNA sequence itself. These resulting changes in gene expression can determine cell fate and tissue development, in turn affecting higher order phenotypes like morphology and behaviour (Jonsson and Jonsson, 2014, 2019; Granada *et al.*, 2018). Importantly, sensitivity of DNAm to both the exogenous and endogenous environment has been well established (explored in Granada *et al.* (2018), Eirin-Lopez and Putnam (2019), or Gavery and Roberts (2017)). Many studies have focused on the prolonged environmental stimuli (Uren Webster *et al.*, 2018; Robinson *et al.*, 2019), however, there is evidence that DNAm changes can be induced even after short exposure time (Marandel *et al.*, 2016), with the nature of environmental stimuli also impacting the phenotypic or physiological outcomes (Moghadam

et al., 2017; Robinson *et al.*, 2019; Beemelmanns, Ribas, *et al.*, 2021; Beemelmanns, Zanuzzo, *et al.*, 2021).

Much of this research into DNAm is focused on the early stages of development (Granada *et al.*, 2018). During this time, genomic patterns of DNAm undergo reprogramming, whereby they are erased post-fertilisation, allowing cellular totipotency, before their re-establishment, to direct cell differentiation and development (Clark and Rager, 2020). Such plasticity is important when organisms inhabit variable environments and helps to produce phenotypes that best enable later life success (Metzger and Schulte, 2016; Eirin-Lopez and Putnam, 2019). Equally, changes to somatic cells DNAm can occur in response to environmental exposure later in life (Granada *et al.*, 2018), with DNAm changes also implicated in post-embryonic phenotypic development (Morán and Pérez-figueroa, 2011; Morán *et al.*, 2013; Baerwald *et al.*, 2016; Gavery *et al.*, 2019).

The methylome is however, dynamic, with changes lost unless maintained throughout the cell cycle. Consequently, there has been variation in the reported length of time that epigenetic signals have persisted in the genome. For example, some have recorded changes that diminish with time (Morán *et al.*, 2013) or dissipate once the environmental stimuli were removed (Gavery *et al.*, 2019), whereas others have shown them to be relatively stable (Gavery *et al.*, 2018), or a mixture of transient and fixed alterations (Beemelmanns, Ribas, *et al.*, 2021).

In addition to somatic inheritance, DNAm patterns can be passed on to subsequent generations if they occur in the germline. This transgenerational inheritance is accepted if the epigenomic signature seen in gametes is passed onto the next and further generations in a stable or fixed way. When specifically referring to fish, the detection of epigenomic signatures in the F2 generation should be taken as a sign of transgenerational inheritance, as the F1 generation will have been derived from cells in the parental population (Labbé, Robles and Herraiz, 2017; Best *et al.*, 2018; Clark and Rager, 2020; Roy *et al.*, 2021). For this to occur, however, parental signatures need to avoid two waves of DNAm erasure. While the first occurs during post-fertilisation (as mentioned above), the second takes place during the formation of primordial germ cells early in development, with parental DNAm patterns later re-established during gametogenesis (Labbé, Robles and Herraiz, 2017; Skvortsova *et al.*, 2019; Clark and Rager, 2020). While a sub-set of genes, known as imprinting genes, have been seen

to avoid these rounds of erasure and inherited in a parent-of-origin manner in mammals, research into imprinting genes in fish is less available (Clark and Rager, 2020; Zhang *et al.*, 2023).

Beyond imprinting genes, the benefit of retaining parental epigenomic patterns is less clear but can be advantageous where the parental environment is predictive of that of the offspring (Venney, Wellband and Heath, 2020). While the mechanism for the inheritance of parental epigenome is yet to be fully characterised, in fish, paternal patterns of methylation transmitted in the sperm are somewhat retained. Specifically, as identified in zebrafish (*Danio rerio*), DNAm is rapidly re-programmed after fertilisation, with sperm initially hypermethylated compared to the oocyte in the newly formed embryo. Importantly, however, maternal DNAm patterns undergo remodelling to approximate that of sperm (Pollock, Swihart and Herbert, 1978; Jiang *et al.*, 2013; Labbé, Robles and Herraiez, 2017; Ortega-Recalde *et al.*, 2019; Skvortsova *et al.*, 2019). This highlights the possibility for the inheritance of paternal methylation patterns, with recent studies providing evidence in support (Gavery *et al.*, 2018, 2019; Rodriguez Barreto *et al.*, 2019; Wellband *et al.*, 2021). It should be mentioned that while maternal DNAm patterns are lost during fertilization, they have been investigated as a mechanism for non-additive maternal effects on offspring (Venney *et al.*, 2020; Venney, Wellband and Heath, 2020)

1.4.2.1. Genome-epigenome-environment interactions

Conversely, genomic regulation of the epigenome is also known. This is mainly studied as mutations and single nucleotide polymorphisms (SNP) in key regions that affect levels of DNAm. Such SNPs can circulate within populations and contribute to increased epigenetic variance, inter-individual variability, and strong family affects (Feil and Fraga, 2012; Taudt, Colomé-Tatché and Johannes, 2016; Hu *et al.*, 2021). Specifically, quantitative trait loci identified from the associations between CpG site methylation and SNPs (termed methylation quantitative trait loci, meQTL), as well as formal quantitative analysis revealing genetic variance (i.e. V_g) and heritability estimates of DNAm themselves, have been demonstrated (Venney *et al.*, 2020; Venney, Wellband and Heath, 2020; Hu *et al.*, 2021). Consequently, where environmental stimuli alter or trigger mutations, this provides a genetically driven mechanism for the environment to alter the epigenome (Figure 1-7).

The above primarily occurs through SNPs in DNA sequences underpinning both cis- and trans-regulatory mechanisms (e.g. enhancer regions). There is generally greater understanding of cis-regulatory components, with SNPs in association with methylation variation clustering in enhancer regions (Taudt, Colomé-Tatché and Johannes, 2016; Villicaña and Bell, 2021). Specifically, SNPs can cause alterations to sequence specific binding of proteins, acting either passively, where proteins bind and block methylation changes, or actively, where they recruit and direct the action of methylation associated enzymes (e.g. DNMTs/TETs) (Villicaña and Bell, 2021). In contrast, SNPs in trans-regulatory elements tend to cluster in active chromatin regions. These SNPs can alter methylation by either affecting the expression of global methylation regulators or genes which have downstream effects on methylation of multiple CpG sites. Trans-regulatory SNPs also alter gene expression indirectly, by first acting as a cis-regulator of DNAm of genes with down-stream effects on methylation at multiple CpG sites. Lastly, when brought into closer proximity with target regions due to the 3D organisation of DNA, trans-regulatory SNPs can act in cis capacity to change DNAm of target loci or disrupt formation of structural variants (Villicaña and Bell, 2021). In addition to the action of these regulatory regions, polymorphisms in methylation related genes themselves, transcription factor binding sites, or CpGs, could directly affect patterns of methylation (Feil and Fraga, 2012; Saradalekshmi *et al.*, 2014; Meaney, 2017).

1.4.3.Impacts and applications of DNAm in aquaculture

There is growing interest in harnessing these epigenetic mechanisms within an aquaculture setting (reviewed by Moghadam, Mørkøre and Robinson, 2015; Gavery and Roberts, 2017; Granada *et al.*, 2018; Eirin-Lopez and Putnam, 2019; Roy *et al.*, 2021). Firstly, where key economic traits are under epigenetic regulation and affected by environmental conditions, their expression can be controlled by husbandry manipulation (Gavery and Roberts, 2017; Granada *et al.*, 2018). This can be practically achieved through the concept of priming, where exposure to an environmental stimulus helps guide epigenetic programming and correct development or generates an epigenetic memory which better allows the individual to respond to the same stimulus later in life. This has been explored in terms of developmental programming of eggs or larvae, as well as broodstock conditioning where parental environment is altered in turn affecting either the internal maternal environment of eggs or

passed on to offspring via epigenetic inheritance (Gavery and Roberts, 2017; Roy *et al.*, 2021) (Figure 1-7).

These concepts can also negatively impact the performance of commercial populations. For example, for species which inhabit multiple husbandry environments, such as hatcheries to grow-out farms, initial environmental variables may fail to set appropriate epigenetic patterns which will enable success later in life. Such an idea can be understood as mal-adaptive programming, whereby the early life environment is too dissimilar from that of later life, or does not properly establish early developmental patterns, leading to poorer performance as adults (Le Luyer *et al.*, 2017; Gavery *et al.*, 2018, 2019; Rodriguez Barreto *et al.*, 2019). Equally, as genetic material from broodstock populations is often widely disseminated to a range of production environments, an epigenetic miss-match between parent and offspring could occur. Consequently, there may be increased variability in the phenotypic performance of a selected population (Taudt, Colomé-Tatché and Johannes, 2016).

Support for such hypothesis can be seen in the poorer performance of hatchery produced fish after only a single generation of hatchery rearing (Araki *et al.*, 2008). Considering this, changes to salmonid epigenomes have been reported in response to a range of various environmental variables typically manipulated in a commercial setting, such as temperature (Burgerhout *et al.*, 2017; Metzger and Schulte, 2017; Beemelmans, Ribas, *et al.*, 2021), hypoxia (Kelly *et al.*, 2020; Beemelmans, Ribas, *et al.*, 2021), dietary composition (Craig and Moon, 2013; Morán *et al.*, 2013; Marandel *et al.*, 2016; Veron *et al.*, 2018) and stress (Moghadam *et al.*, 2017; Uren Webster *et al.*, 2018; Robinson *et al.*, 2019). Further evidence is gained from comparisons made between the methylome of wild caught or semi-natural fish with hatchery reared counterparts. These studies have consistently shown populations to diverge genetically by natal origin, but epigenetically by rearing environment (Le Luyer *et al.*, 2017; Gavery *et al.*, 2018). Moreover, evidence from these studies, including parallel epigenetic modifications in distinct populations (Le Luyer *et al.*, 2017), shared DNAm patterns between tissues (Gavery *et al.*, 2018), and similar cohort of effect genes (Wellband *et al.*, 2021), suggest a possible unifying effect of hatchery environment on DNAm.

Due to the genetic regulation of the epigenome, it can become the focus trait of quantitative genetic studies and selection itself, as has been performed in Rainbow trout, *O. mykiss*,

(Venney *et al.*, 2020; Venney, Wellband and Heath, 2020) and Three-spined stickleback, *Gasterosteus aculeatus*, (Hu *et al.*, 2021), as well as GxE in DName patterns investigated, as demonstrated in transgenic Coho salmon, *Oncorhynchus kisutch*, (Christensen *et al.*, 2021). However, epigenetic variation can also have negative implications for breeding programs (Moghadam, Mørkøre and Robinson, 2015; Gavery and Roberts, 2017; Roy *et al.*, 2021). Specifically, it can incorrectly appear heritable because of associations between a SNP and epimutation- understood as a heritable change in epigenetic gene expression regulation not associated with a change in the DNA sequence itself (Taudt, Colomé-Tatché and Johannes, 2016). This can form from environmental stimuli triggering an epimutation at a particular SNP. However, as this epimutation is not itself stably or reliably inherited between generations, it can bias EBV or genetic variance (Moghadam, Mørkøre and Robinson, 2015). In the absence of environmental stimuli, these epimutations can also occur stochastically, all of which complicates breeding programs when based on genetics alone (Taudt, Colomé-Tatché and Johannes, 2016).

While this is true for all breeding programs, those employed in aquaculture are often comparatively simple, limiting the ability to disentangle genomic from epigenomic polymorphisms. However, suggestions of how to account for this have been purposed. In brief, this involves correcting additive genetic variance of a locus by accounting for an epigenomic component, itself consisting of (1) paternal and/or maternal inherited effects, (2) sex-specific effects and (3) gene by epigenome interactions (Moghadam, Mørkøre and Robinson, 2015). Quantitative applications then apply this theory across the genome, though such approach would require deep genomic and epigenomic information (Moghadam, Mørkøre and Robinson, 2015; Gavery and Roberts, 2017).

While this describes non-heritable epimutations, heritable epimutations are also possible, as has been demonstrated most clearly in aquatic plants (Taudt, Colomé-Tatché and Johannes, 2016). Over time an epimutation can become unattached from the underlying DNA sequence, leading to epialleles that segregate in the genome independently (Taudt, Colomé-Tatché and Johannes, 2016). Therefore, if sufficient epigenomic information is available, quantitative genetic approaches can be applied to investigate the epigenetic architecture of traits, as well

as applying epigenomic selection (Taudt, Colomé-Tatché and Johannes, 2016; Gavary and Roberts, 2017).

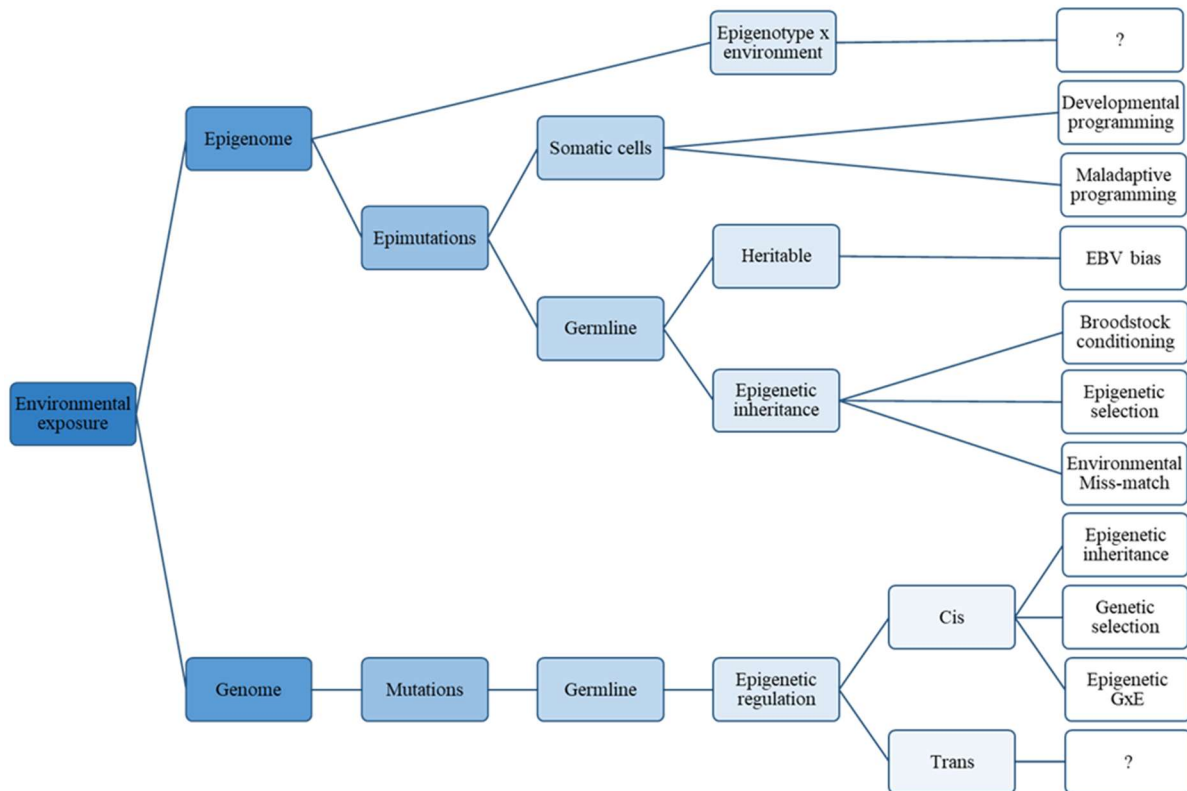


Figure 1-7 Pathways for the environment to alter the epigenome from a genetic and epigenetic perspective, leading to their applications or considerations in aquaculture. Unclear aquacultural implications indicated by a question mark. Figure inspired/based on those presented in Taudt, Colomé-Tatché and Johannes (2016) and Bollati and Baccarelli (2010), as well as information in Taudt, Colomé-Tatché and Johannes (2016).

1.5. Knowledge gap, and research objectives

Employment of intensive land-based recirculating systems, such as RAS, is now recommended to sustain a continual increase in productivity as they allow greater environmental control and increased stocking density while mitigating some of the industry's environmental impact (Clarke and Bostock, 2017). Despite this, in Scotland, the predominant FW culture system remains floating net-pens in lochs up to smoltification. In contrast to RAS, loch systems are relatively easy to set up and operate as well as carrying a lower economic investment. However, these two systems differ in many of their environmental characteristics. Namely RAS are distinct in terms of stocking density, temperature, light quality and quantity, water parameter variables and expected stability, whereas loch systems are more variable, have more limited husbandry regulation, and are exposed to ambient environmental

conditions (Ebeling and Timmons, 2012; Good and Davidson, 2016; Clarke and Bostock, 2017; Schumann and Brinker, 2020).

Anecdotal evidence from the industry has suggested that RAS-reared Atlantic salmon performance post-saltwater transfer is poorer than loch-reared counterparts. More recently, studies have shown that RAS-reared fish show differences in terms of physiological and allometric parameters which have been linked to poorer SW growth and survival (e.g. Lai *et al.*, 2024), but empirical evidence as to why this may be occurring is relatively limited. Given the role the environment plays in Atlantic salmon FW development, it is theorised the RAS environment may not appropriately prepare parr for their SW transfer and later life successes. For the industry to increase the use of RAS, understanding the phenotypic, genomic and epigenetic impact of the RAS environment is needed. Therefore, the present thesis plans to bridge this knowledge gap by investigating (1) genotype-environment interactions between RAS and loch-reared commercial population and (2) DNA methylation changes to the RAS specific environment. This will help answer whether good performance in the RAS environment is related to good performance in loch, if this is predictive of performance in SW, and lastly if this is underpinned by maladaptive programming of the epigenome. Specifically, the research aims are as follows:

(1) to investigate the comparative performance of current commercial stocks in RAS to help inform future breeding decisions (**Chapter 2**),

(2) to study the impacts of environmental variables differing between FW-rearing systems on DNA methylation and gene expression patterns (**Chapter 3**), and

(3) to assess the impact of RAS-rearing on Atlantic salmon SW performance, through assessment of genotype-environment interactions (**Chapter 4**).

Chapter 2-Genotype-environment interactions in Atlantic Salmon, *Salmo salar*, Reared in Freshwater Recirculating and Loch System

This Chapter has been adapted from the research paper published by Evolutionary Applications as:

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I was involved in the methodology, formal analysis at all stages, investigation and writing of the original publication manuscript. Further contributions were as follows; **M.B.:** Validation, Data Curation, Investigation, Supervision, Writing-Review & Editing; **A.B.G.:** Methodology, Data Curation; **S.A.:** Methodology, Data Curation; **R.D.H.:** Conceptualisation, Project administration; **A.D-W.:** Conceptualisation, Project administration; **A.N.:** Conceptualisation, Resources; **H.M.:** Resources, Conceptualisation, Supervision, Funding acquisition, Writing-Review & Editing; **A.P.G.:** Supervision, Project administration, Writing-Review & Editing.

2.1.Abstract

There is increasing interest in use of recirculating aquaculture systems (RAS) as they permit increases in productivity and husbandry control while reducing environmental impacts and water use. However, in some regions there is still wide use of other culture systems, due to their relatively low cost and ease of running, and selective breeding programs will likely have been based on the performance of fish in these environments. It is therefore of interest to investigate the comparative performance of current stocks in RAS to help inform future breeding decisions. Working within a commercial setting, this study aimed to investigate the genetic architecture of phenotypic traits, quantify genotype-environment interactions (GxE) of salmon smolts, and compare genetic markers associated with phenotypic expression in freshwater lochs or RAS. Here, parr were co-reared before being evenly split between a freshwater net-pen or recirculating system. After ~eight weeks, 1,000 fish were sampled per environment and genotyped to ~65K SNPs, allowing estimates of genetic and phenotypic parameters, as well as quantification of GxE as the genetic correlation between traits measured in each environment (r_{gxe}). Results indicated RAS-rearing produced smaller fish in terms of weight and length but showed greater condition factor and uniformity. A significantly smaller component of residual variance was found in RAS, translating into comparatively greater heritability estimates. Low GxE was found for length ($r_{gxe}=0.778$, s.e 0.15) and condition factor ($r_{gxe}=0.853$, s.e 0.17), whereas significant re-ranking was identified for whole-body weight ($r_{gxe}=0.624$, s.e 0.14), and environment-specific differences in the trait associations were identified. In particular, a region on chromosome 22 was found in association with condition factor in the RAS population only. This suggests that if employment of RAS continues to increase, the performance of current commercial populations may be below what could be achieved if environment-specific breeding programs are not established. However, such conclusions are based on statistical results and further investigation in use of RAS for current populations is necessary.

2.2.Introduction

In Scotland, the dominant salmon producer within the UK, use of floating net-pens in freshwater (FW) lochs are still primarily used for rearing Atlantic salmon (*Salmo salar* L. 1758) up to smoltification (Bergheim *et al.*, 2009; Regan *et al.*, 2021). However, over recent decades there has been a progressive uptake of land-based systems, in particular fully closed recirculating aquacultural systems (RAS), for commercial production (Bergheim *et al.*, 2009), in part motivated by the advantages this system offers (Thorarensen and Farrell, 2011; Ebeling and Timmons, 2012).

However, re-use of water through recirculating systems requires mechanical, chemical, and biological water filtration to prevent water chemistry changes during fish rearing (Ebeling and Timmons, 2012). If not monitored and controlled the accumulation of certain substances can affect fish physiology and welfare (Fivelstad, 2013; Gorle *et al.*, 2018; Kocour Kroupová *et al.*, 2018; Schumann and Brinker, 2020). Consequently, both the set up and operation of RAS carries a high economic cost, often leading to rearing fish at higher densities to maintain profitably (Thorarensen and Farrell, 2011; Liu *et al.*, 2016). In contrast to RAS, traditional loch-based systems are cost effective and simpler to operate, but they allow limited environmental control and are exposed to external factors (Ellis *et al.*, 2016). Furthermore, current commercially used salmon lines will have undergone selection based on their performance in loch net-pens during their FW phase. Therefore, for the increased adoption of RAS it is pertinent to assess relative performance of these selected stocks across both environments.

One strategy to compare the performance of different families across various environments is through exploration of genotype-environment interactions (GxE), defined as the mean phenotypic change of a given genotype in different environments (Falconer, 1952; Falconer and Mackay, 1996). As explained in the General Introduction, Chapter 1.3.1, GxE can be identified as heterogeneity of genetic parameters when compared between environments (Sae-Lim *et al.*, 2014). Of particular concern for aquaculture is, however, re-ranking, whereby the best performing genotype in one environment does not perform equally well in another (Mulder *et al.*, 2006; Sae-Lim *et al.*, 2016). This is typically quantified as the genetic correlation between the same trait measured in different environments (r_{gxe}) (Mulder *et al.*, 2006; Sae-Lim *et al.*, 2016).

While different thresholds have been suggested for this value (Robertson, 1959; Navarro *et al.*, 2009b), a recent benchmark of 0.7 has been suggested for aquacultural species (Sae-Lim, 2013). Correlations falling below this suggest the presence of significant GxE, inferring selective gains will not be equally realised across environments, reducing the efficiency of breeding programs (Mulder and Bijma, 2005; Mulder *et al.*, 2006). Estimates returned in other species comparing their comparative performance in RAS to other systems have generally returned significant estimates of GxE, with genetic correlations ranging from 0.27 to 0.65 (Mas-Muñoz *et al.*, 2013; Sae-Lim *et al.*, 2014; Fernandes *et al.*, 2019; Li *et al.*, 2019; Van Sang *et al.*, 2020) though weaker GxE have been reported by others (Dupont-Nivet *et al.*, 2008, 2010; Turra *et al.*, 2016). Similar studies between FW environments employed in Atlantic salmon aquaculture are yet to be performed.

This present study aims to bridge this gap by investigating the genotype-environment interactions between the RAS and loch environment in Atlantic salmon, assessed during their FW development and within a commercial context. The objectives include: (1) estimating phenotypic and genetic parameters for Atlantic salmon within each rearing environment, (2) determining GxE between FW-rearing environments by estimating r_{gxe} , and (3) comparing genetic markers associated with trait expression between environments. The insights for this research will inform on how genetic stocks perform under different environmental conditions and will be instrumental in guiding future breeding strategies and husbandry decisions within the context of the growing utilisation of RAS.

2.3.Materials and Methods

2.3.1. Ethics

Animal handling and collection in this study was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 Amended Regulations (SI2012/3039) and the work was approved by the University of Stirling Animal Welfare and Ethics Review Board (AWERB202148713775). This project was also part of a larger BBSRC/NERC funded project, ROBUSTSMOLT, which also received ethical approval (Comparative RAS Study 2585).

2.3.2.Source population

The study population came from the nucleus family breeding programme of MOWI Ireland 2022 Generation, produced from ~163 dams and 90 sires. For the study population, broodstock were spawned over two subsequent days. Families were produced mostly using a hierarchical mating structure, where one male was used to inseminate the eggs of two females. Breeding goals for this nucleus population focused on: resistance to cardiomyopathy syndrome (CMS), growth (measured as body weight), and lower sexual maturity. Eggs were extracted from sacrificed dams using air pressure. There was no hormonal induction of spawning or maturation.

2.3.3.Experimental design

From the source population, in January 2021 ~250,000 eyed eggs were transferred to recirculating aquaculture facilities (RAS) in the northwest of Scotland (Inchmore). Eggs were incubated separately until hatching (~400 degree days), after which they were combined into groups, maintaining the same number per dam. From first feeding fish were kept in a single fry unit for ~ five weeks before being split between two fry tanks (~116,500 fry/tank). After three weeks, the fish from both fry tanks were reallocated across four fry tanks, before the number of fish in each tank was approximately evened out to ~52,000. After a further six weeks the population was transferred into two smolt tanks (~96,500 in each) at which point a bottom ~5% cull was performed which removed 11,197 fish. Remaining fish from the two smolt tanks underwent vaccination over two consecutive days. The population was then placed back into 10 fry tanks (~19,000 each) with a further bottom ~4.7% cull performed, removing 8,961 fish.

In September 2021, before approximately half the population was transferred to FW loch site for the smolting (Loch Garry), fish were sexed and split by phenotypic sex via use of ultrasound. In the middle of sexing and as soon as there were enough males and females for transportation, three tanks of males and three tanks of females were moved and pooled into one net-pen by phenotypic sex at the FW loch site (such that there was a single pen per phenotypic sex). This corresponded to 91,708 fish (32,584 females and 59,124 males). The remaining population in the RAS facilities included 91,713 fish (45,874 females and 45,839

males) split between six fry tanks -three male and three female- with ~15,000 smolts in each. Fish remained in these environments until November 2021. In RAS facilities, tanks were ~50m³, while in the loch environment, pens had a capacity of ~2,048 m³. This corresponded to a density of ~306 fish/ m³ and ~22 fish/ m³ in RAS and loch environments, respectively. In RAS, parr were initially held under 12~h light/darkness (LND) cycles. After ~one month, the population remaining in RAS was placed under continuous light (24~h light, LL). In the population referred to as FW loch, parr were held under winter (without light) photoperiod before exposure to usual 400 degree days of light (LL) prior to going to sea. In commercial systems, parr are often exposed to continuous light (LL) as it enhances growth performance. However, for parr to undergo smoltification, they require exposure to a winter short day (LND) photoperiod followed by a return to an increasing or long day photoperiod (LL) (Björnsson *et al.*, 2000). Both populations were fed the same diet (MOWI Proteus). Fish remained in these FW environments for approximately eight weeks (56 days), until November 2021.

In both environments, measurements of water temperature (°C), pH, and oxygen saturation (%) were taken daily. Additional water quality parameters were recorded in the RAS environment including: carbon dioxide (CO₂), chloride (Cl), total ammonia and nitrate (TAN), ammonia (NH₃), nitrite (NO⁻²), nitrate (NO⁻³) concentration (mg/L), as well as measurements of total alkalinity (mg CaCO₃/L), hardness (mg CaCO₃/L), conductivity (siemens per meter, sm), turbidity (turbidimetric turbidity unit, NUT), feed fed (kg), new water added (m³/day), and salinity (parts per million, ppt). For each variable, across the period of sperate rearing, its average and standard deviation (s.d) were calculated.

2.3.4.Sample collection and phenotypic traits

In November 2021, a total of 1,000 smolts were sampled per FW-rearing environment, when the population was approximately 10-months of age (~104.85g (s.d 28.50)). Sampling occurred by random netting of fish from tanks/pens. In the loch environment as there was only a single pen per phenotypic sex fish were sampled from both. In the RAS environment, the phenotypic male and female fish were split across three tanks for each. Due to site restrictions, fish were sampled from only one tank per sex. The number of fish removed from tanks/pens was unequal between sexes due to biomass limitations known at the SW site which meant only the

phenotypically female fish would be able to be maintained separately based on their FW-rearing environment. As this population will also be sampled at SW (see Chapter 4), there was a ¾ skew towards phenotypically female fish at the FW sampling. This was with the aim of maximising the number of families and genetic variability captured in the FW stage, allowing downstream analysis of GxE between FW and SW populations, all the while still allowing investigation into sex effects in FW. In both environments, smolts were sampled over two consecutive days.

Measurements of whole-body weight (WBW) and length (tip of head, snout, to the deepest point of the fork in the caudal fin) were recorded, from which Fulton's condition factor (K) was calculated as $K = 100 * (W_{\text{grams}}/L_{\text{cm}}^3)$ (Fulton, 1904). Additionally, fin clips were taken from the adipose fin of all sampled fish and stored in 2ml of 95% ethanol until genotyping.

2.3.5. Genotyping and quality control

Collaborators at MOWI made available parental (broodstock) SNPs previously genotyped on non-public NOFSAL03 (~55K) SNP array developed by Nofima, MOWI Genetics and Benchmark Genetics. Fin clips sampled here were genotyped by IdentiGEN Ltd on a custom ~66K ThermoFisher SNP array developed internally by MOWI Genetics AS (non-public Axiom array, SALMOWI) and based on NOFSAL03. SNPs were called based on major allele frequency, following standard genotyping pipeline for polyploidy and using Applied Biosystems -- Analysis Power Tools (APT) v2.11.6 (ThermoFisher Scientific, 2024). Genotypic sex was assigned based on intensity scores of public markers for sex-determining region Y gene (SDY).

Quality control (QC) of genotypes was performed with PLINK v1.9 (Purcell *et al.*, 2007). Firstly, unaligned SNPs (i.e. those not aligned to chromosomes 1-29) and those with more than one SNP recorded at that genomic position (using PLINK function *--list-duplicate-vars*) were excluded. Further filtering steps were as follows: individuals who had more than 10% of their genotypes missing, SNPs that were missing in more than 10% of individuals' genotypes, SNPs that failed to meet Hardy-Weinberg equilibrium ($P\text{-value} < 10^{-6}$), and SNPs with minor allele frequency lower than 0.005 were all removed. Common SNPs to both parental and offspring populations were identified from the overlap in the online Venn Diagrams resource at

BioTools.Fr (<https://www.biotoools.fr/misc/venny>) and retained for further analysis. A list of these common SNPs (extracted using PLINK function *--extract,*) was used to merge the two populations (*--bmerge*, PLINK function). Filtered genotypes were formatted into .txt file using *genio* (Ochoa, 2023) package in R.

2.3.6. Pedigree reconstruction and population structure

Pedigree reconstruction and family assignment was performed by MOWI using the sequenced genotypes (as described above) and their own in-house software which employs the opposite homozygosity (OH) method. Specifically, between all the sires and dams mated to produce the study population, the OH was counted when the broodstock genotypes were compared to that of each of the sampled offspring. Sire and dams with the lowest OH were assigned temporarily as a parent. Sire and dam combinations based on OH were then compared to the list of known matings recorded by MOWI. When sires and dams did not appear in the know mate pairings, the parentage was rejected, and a likelihood approach was used for those offspring.

Genomic relationship matrix (GRM) between individuals of the population was computed using GCTA software (Yang *et al.*, 2011). Principal component analysis (PCA) of the GRM was then generated, with the first two components visualised *ggplot2* (v3.4.2) (Wickham, 2011) in R v4.2.2 (R Core Team, 2022). The number and size of full-sib families were calculated for both populations. The difference between family size in each environment was tested with Chi-square test for association, at 5% significance. The number of shared families between populations was investigated, and only fish belonging in these shared families were retained for the downstream analysis.

2.3.7. Statistical analysis

Normality was assessed from histograms of raw phenotypic data and q-q plots post statistical testing. Fish with missing phenotypic data were removed. Mean and standard deviation (s.d) were calculated for each trait overall, and within each FW environment. Effects of genotypic sex and environment (RAS and Loch), with nested effect of tank or pen within environment, on trait averages and variance were investigated through two-way ANOVA (Type II

sequential) with Post Hoc Tukey test (P-value<0.05). All statistics were evaluated with R v4.2.2 (R Core Team, 2022).

2.3.8. Within environment genetic parameters

All models and genomic analysis were performed in BLUPF90 software (Miszta *et al.*, 2002).

2.3.8.1. Univariate analysis

For both the RAS and loch specific populations univariate animal models (Eq. 2-1) were fit with variance components estimated via implementation of a restricted maximum likelihood (REML) approach as follows,

$$\mathbf{y} = \boldsymbol{\mu} + \mathbf{X}\mathbf{b} + \mathbf{Z}\mathbf{u} + \mathbf{e} \quad \text{Eq. 2-1}$$

where, $\mathbf{y}$ was a vector of phenotypic records and $\boldsymbol{\mu}$ was the overall population mean, $\mathbf{X}$ a design matrix linking individuals to a vector of fixed effects $\mathbf{b}$. Specifically, for both environments, genotypic sex (2 levels, M=1, F=2) and tank/pen (2 levels) were included as fixed effects in $\mathbf{b}$. Genotypic sex was fitted rather than phenotypic sex as the two were not found to be consistent with one another (Appendix A. 1), particularly in the RAS environment. Furthermore, as the population was split by phenotypic sex between tanks/pens, the separate effect of tanks/pen and phenotypic sex would be confounded.

$\mathbf{Z}$ was a design matrix linking individuals to a vector of additive genetic effects $\mathbf{u}$. Analysis was firstly performed when $\mathbf{u}$ was estimated from the pedigree (A matrix), in which $\mathbf{u}$ had a multivariate normal (MVN) distribution $\sim \text{MVN}(0, \mathbf{V}_g * \mathbf{A})$, where $\mathbf{A}$ was the numerator relationship matrix and $\mathbf{V}_g$ was additive genetic variance. Secondly, analysis was performed where parameters were estimated using the genomic (SNP) data, where $\mathbf{u} \sim \text{MVN}(0, \mathbf{V}_g * \mathbf{GRM})$, and $\mathbf{GRM}$ was the genomic relationship matrix. Additionally, the analysis was repeated implementing single-step genomic evaluation where $\mathbf{u} \sim \text{MVN}(0, \mathbf{V}_g * \mathbf{H})$. In this the $\mathbf{H}$ matrix combined information from both pedigree and SNP data, as defined in Legarra *et al* (2014).

Lastly, $\mathbf{e}$ was a vector of residual effects with $\sim \text{MVN}(0, \mathbf{V}_r * \mathbf{I})$, where $\mathbf{I}$ was an identity matrix and $\mathbf{V}_r$ was residual variance. For analysis, full pedigree and SNP data was provided but using only the phenotypic data of a trait when expressed in a specific environment (RAS or loch).

For each trait (WBW, length, K), narrow sense heritability ($h^2 = V_g/V_p$), coefficient of phenotypic variance ($CV = (SD_p/\bar{X}) * 100$, where $\bar{X}$ is the phenotypic mean) and coefficient of genetic variance ($CGV = (\sqrt{V_g}/\bar{X}) * 100$) were estimated (Cheung, 2020).

2.3.8.2. Within environment correlations

Bi-variant models were subsequently fit (Eq. 2-2) between pairwise combination of traits within each environment, corresponding to y_1 and y_2 :

$$\begin{pmatrix} y_1 \\ y_2 \end{pmatrix} = \begin{pmatrix} x_1 & 0 \\ 0 & x_2 \end{pmatrix} \begin{pmatrix} b_1 \\ b_2 \end{pmatrix} + \begin{pmatrix} z_1 & 0 \\ 0 & z_2 \end{pmatrix} \begin{pmatrix} u_1 \\ u_2 \end{pmatrix} + \begin{pmatrix} e_1 \\ e_2 \end{pmatrix} \quad \text{Eq. 2-2}$$

In this model, identity matrix as well as fixed and random effects were the same as those in Eq 2.1. Estimated genetic (co)variances and V_g , had the structure $\begin{pmatrix} V_{g,1} & \text{Cov}(1,2)_g \\ \text{Cov}(2,1)_g & V_{g,2} \end{pmatrix}$. The genetic correlation between traits was calculated as $r_g = \frac{\text{Cov}(1,2)_g}{\sqrt{V_{g,1} * V_{g,2}}}$. Residual (co)variances had the structure $\begin{pmatrix} V_{r,1} & \text{Cov}(1,2)_r \\ \text{Cov}(2,1)_r & V_{r,2} \end{pmatrix}$ and residual correlation between traits calculated as $r_r = \frac{\text{Cov}(1,2)_r}{\sqrt{V_{r,1} * V_{r,2}}}$.

2.3.9. Genotype-environment interactions

GxE were estimated using a multi-trait approach in which same trait measured in different environments was treated as two independent traits. The strength of GxE was then quantified by the genetic correlation (r_{gxe}) between the two traits (Mulder and Bijma, 2005; Sae-Lim *et al.*, 2014). This was performed by fitting a model as Eq.2-2, where y_1 and y_2 would, for example, correspond to WBW in RAS and loch respectively. Random genetic effects were fitted as in uni- and bi-variant analyses. Fixed effects of tank, genotypic sex in RAS and genotypic sex in loch were included. Residual covariance was set to zero as each fish could only inhabit in a single environment (Eq. 2-3).

$$\begin{pmatrix} e_1 \\ e_2 \end{pmatrix} \sim \text{MVN} \left(I * \begin{pmatrix} \sigma_{r,1}^2 & 0 \\ 0 & \sigma_{r,2}^2 \end{pmatrix} \right) \quad \text{Eq. 2-3}$$

2.3.10. GWAS

To characterise environmental differences in the genetic regulation of phenotypic traits, a test for genome wide association (GWAS) was performed on environment-specific traits,

following pipeline by Roslin Aquaculture (2023). Using GCTA software (Yang *et al.*, 2011), a mixed linear model (MLM) approach, following a leave one chromosome out principle (LOCO), was fitted with the same fixed and random effects as Eq. 2-1. This was performed using the same set of filtered SNPs (Appendix A. 3) as both univariant and bivariate analysis. In MLM-LOCO approach, for a given SNP, information on the chromosome its located is excluded from the calculation of **GRM**. This approach can help reduce bias and false positives, while increasing power and accuracy (Yang *et al.*, 2014). Lambda (λ) -which indicate if test statistics have been inflated - was calculated for each test as $\text{median}(\chi^2)/\text{expected median}(\chi^2)$ (Yang *et al.*, 2011).

A 5% significant threshold was calculated using Bonferroni correction at both the genome and chromosome level. This was based of the total number of filtered SNPs ($-\text{Log}_{10} * (0.05/\text{Total SNP number})$) as well as the average number of SNPs per chromosome ($-\text{Log}_{10} * (0.05/(\text{Total SNP number}/\text{Chromosome number}))$). Data visualization was performed in R (version 4.2.2) (R Core Team, 2022) using ggplot2 (Wickham, 2011) and following workflow as described by Holtz (no date). The genomic location of significant SNPs was identified in *Salmo salar* reference genome (Ssal_v3.1, RefSeq GCF_905237065.1) using UNCS genomic browser (<http://genome.ucsc.edu>). The overlapping NCBI (<https://www.ncbi.nlm.nih.gov/>) or Ensembl (<https://www.ensembl.org>) gene ID then was recorded. If not overlapping a gene, the nearest was identified.

2.4.Results

2.4.1.Water quality parameters

The main environmental differences between the RAS and loch systems can be seen in Figure 1-2. Mean water temperature in RAS was 13.21°C (s.d 0.93), greater but more constant in comparison to the loch environment (10.86°C (s.d 2.02)), where the latter showed a progressive decrease with time. Oxygen saturation was greater in RAS (101.09% (s.d 2.62)) compared to loch (90.02% (s.d 0.57)), but the latter showed less variability. Lastly, pH was more acidic in the loch environment (6.30 (s.d 0.09)) compared to RAS (6.96 (s.d 0.16)) but showed lower variance in the loch. Additional water quality parameters were recorded in RAS and available in Appendix A (A. 2).

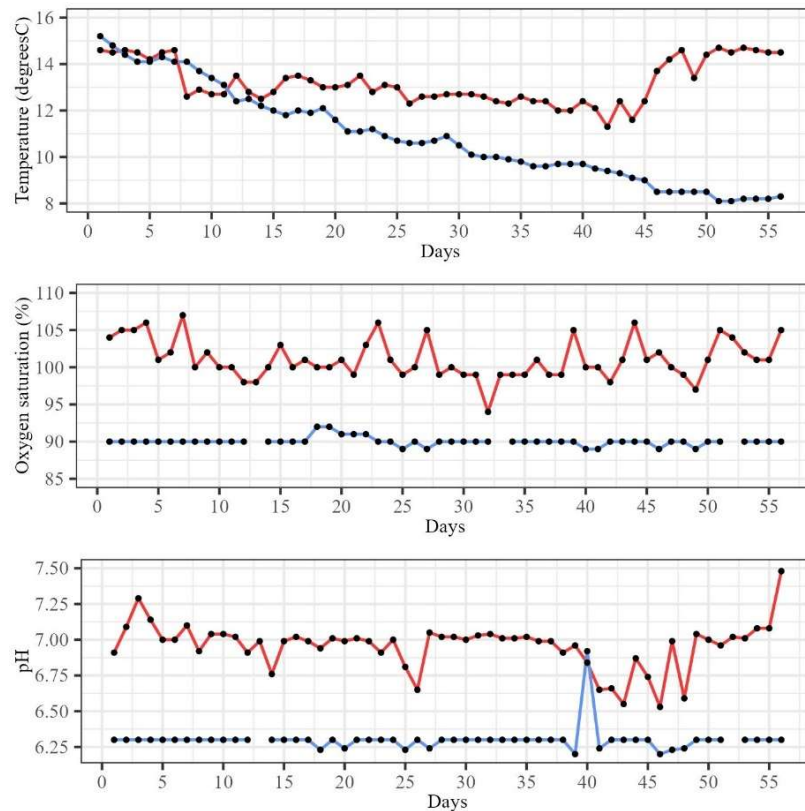


Figure 2-1 Temperature (°C), oxygen saturation (%), and pH for both the RAS (red) and loch (blue) environment over the 56 days of separate freshwater-rearing

2.4.2. Genotyping and population structure

In both environments mortality rate was low. The number of mortalities in the RAS environment was 2,657 corresponding to 2.90%. In the loch environment this value was only slightly greater at 3,836 mortalities, corresponding to a loss of 4.18%.

A total of 2,000 fish were sampled. Genotypic data was obtained for 1,942 offspring. Based on filtered SNPs and GRM, there was little indication of population structure within the parental and offspring population (Appendix A. 12). Genotyping returned a total of 55,357 and 65,774 SNPs for parent and offspring fish respectively. Post-filtering, 53,489 and 59,578 SNPs were retained for parents and offspring, respectively (Appendix A. 3). Of these two filtered sets of SNPs, 45,751 that were common to both parents and offspring and retained for further analysis. Distribution of the final set of SNPs can be seen in Appendix A (A. 4).

Based on pedigree reconstruction sampled fish were assigned to 72 sires and 139 dams in the parental population. 65 offspring could not be assigned to a sire. Of these 41 also failed to be assigned to a dam. From these, 141 full-sib families (including two families with unsigned

sire), 72 sire, and 139 dam families were identified of which 134, 71 and 134, respectively, were shared between environments. Additionally, due to missing measurements of whole-body weight (WBW) two fish were removed, leaving a total of 1940 fish. Categorical breakdown of the population between genotypic sexes and environments can be seen in Table 2-1.

Table 2-1 Categorical split of fish retained for downstream analysis. Split by freshwater-rearing environment, tank/pen and genotypic sex.

	RAS		Total	Loch		Total	Overall
	Tank 1	Tank 2		Pen 1	Pen 2		
<i>Male</i>	473	190	663	201	191	392	1055
<i>Female</i>	259	54	313	518	54	572	885
<i>Total</i>	732	244	976	719	245	964	1940

Though the average full-sib family size was similar between FW environments (6.8 and 6.9 in the RAS and loch environments, respectively), this was variable and ranged from 1 to 28. Furthermore, for each full-sib family, its size was significantly different between environments (Chi-squared statistic = 308.59, degrees of freedom = 132, P-value = 3.56×10^{-16}), with the (absolute) difference in family size between environments ranging from 14 to 18.

2.4.3. Phenotypic parameters

Data visually approximated normal (Appendix A. 13-A. 14). On average, whole-body weight (WBW) was 104.85g (s.d 28.50), length 20.79cm (s.d 1.87) and condition factor (K) 1.25 (s.d 0.10). FW-rearing environment had a significant effect on WBW (degrees of freedom = 1/1934, F-value = 1079.843, P-value < 2×10^{-16}), length (degrees of freedom = 1/1934, F-value = 1244.284, P-value < 2×10^{-16}), and condition factor (degrees of freedom = 1/1934, F-value = 24.028, P-value = 1.03×10^{-6}). Specifically, while fish reared in the loch were heavier and longer than RAS counterparts, they had a lower condition factor (Table 2-2, Appendix A. 13).

Sex also had a significant effect on WBW (degrees of freedom = 1/1934, F-value = 7.56, P-value = 6.03×10^{-3}) and length (degrees of freedom = 1/1934, F-value = 13.15, P-value = 2.96×10^{-4}), while sex-by-environment interaction did not (WBW: degrees of freedom = 1/1934, F-value = 0.62, P-value = 0.43, length: degrees of freedom = 1/1934, F-value = 0.142, P-value = 0.71). Specifically, overall females were on average heavier (108.42g, s.d 29.65) and longer (20.14, s.d 1.94) than males (WBW: 101.85g, s.d 27.14, length: 20.01, s.d 1.79), but within FW environments, males were larger. While this was true for both WBW and length, the difference in sex performance

was significant only for length in the loch environment (p -value=0.02). Furthermore, there was no effect of sex (degrees of freedom =1/1934, F -value=3.510, P -value=6.11e⁻²) or sex-by-environment interaction on condition factor (degrees of freedom=1/1934, F -value=1.02, P -value=0.31). Notably, the nested effect of tank within environment also significantly impacted WBW (degrees of freedom=2/1934, F -value=19.17, P -value=5.73e⁻⁹), length (degrees of freedom=2/1934, F -value=46.93, P -value<2e⁻¹⁶), and condition factor (degrees of freedom=2/1934, F -value=49.36, P -value P -value<2e⁻¹⁶). A difference in performance was more apparent between tanks in the RAS than in net-pens in the loch environment.

Across traits, loch-reared fish exhibited consistently higher standard deviation and coefficient of variance than RAS fish (Table 2-2).

2.4.4.Genetic parameters

While results from models using the H matrix are reported in full here (Table 2-2, Table 2-3), estimates of genetic parameters from A matrix and GRM are available in Appendix A (A.5-A.8). In brief, similar estimates for genetic parameters were obtained from models fitting H matrix or GRM while outputs from A matrix were lower and showed greater variability. For H matrix, 0.67% of genotypes were missing. Correlation between diagonal elements of GRM and A matrix was 0.57. Correlation of off diagonal elements was slightly below the 0.5 threshold (as suggested by software employed) at 0.43 suggesting a possible mismatch between pedigree and genomic information.

2.4.4.1.Genetic variance and trait heritability

In all traits, residual variance was significantly greater in the loch environment (Table 2-2). While WBW estimates of genetic variance were similar between environments, for length and condition factor genetic variance was greater in the RAS environment. This translated into significantly greater heritability estimates in the RAS compared to loch environment. Specifically, across traits, heritability estimates were moderate and low for the RAS and loch-reared populations respectively. Equally, coefficient of genetic variance was greater for the RAS population across traits.

Table 2-2 Descriptive statistics for environment-specific populations, where WBW is whole-body weight , K is condition factor, CV the coefficient of variation, V_r the component of residual variance, V_g the component of genetic variance, CGV the coefficient of genetic variance, h^2 narrow sense heritability and s.e standard error.

	WBW (g)		Length (cm)		K	
	RAS	Loch	RAS	Loch	RAS	Loch
<i>Mean (s.d)</i>	88.03 (17.87)	121.87 (27.11)	19.05 (1.25)	21.35 (1.67)	1.26 (0.08)	1.24 (0.11)
<i>CV %</i>	20.30	22.24	6.55	7.83	6.67	8.80
<i>V_r (s.e)</i>	175.08 (13.26)	591.94 (35.11)	0.83 (0.62e ⁻¹)	2.33 (0.14)	0.38e ⁻² (0.27e ⁻³)	0.10e ⁻¹ (0.59e ⁻³)
<i>V_g (s.e)</i>	127.1 (21.17)	129.58 (33.26)	0.55 (0.95e ⁻¹)	0.43 (0.12)	0.22e ⁻² (0.39e ⁻³)	0.15e ⁻² (0.50e ⁻³)
<i>CGV %</i>	12.81	9.34	3.91	3.06	3.76	3.15
<i>h^2 (s.e)</i>	0.42 (0.55e ⁻¹)	0.18 (0.43e ⁻¹)	0.40 (0.55e ⁻¹)	0.15 (0.43e ⁻¹)	0.37 (0.52e ⁻¹)	0.13 (0.42e ⁻¹)

2.4.4.2. Within environment genetic correlations

Genetic and residual correlations between traits within each environment are shown in Table 2-3. Significantly positive genetic correlations were seen between WBW and length in both environments. Positive genetic correlations were also seen between WBW and condition factor, though this was greater in the RAS environment. Significant negative genetic correlations were found between length and condition factor in the loch environment. In contrast a positive correlation was seen between the two in the RAS environment, though the standard error did span zero. Across environments, residual correlations between traits were similar. WBW and length were strongly positively correlated, a non-significant positive correlation was recorded for WBW and condition factor, and non-significant negative correlation recorded for length and condition factor.

Table 2-3 The genetic (r_g), residual (r_r) and phenotypic (r_p) correlations between phenotypic traits within RAS and loch environments. WBW stand for whole-body weight (g), K is condition factor, s.e is standard error. Values different from one (based on if s.e spans zero) are indicated in bold.

Environment		r_g (s.e)	r_r (s.e)	r_p (s.e)
<i>WBW-Length</i>	RAS	0.95 (0.55e ⁻¹)	0.94 (0.58e ⁻²)	0.95 (0.41e ⁻²)
	Loch	0.94 (0.36e ⁻¹)	0.92 (0.66e ⁻¹)	0.92 (0.51e ⁻²)
<i>WBW-K</i>	RAS	0.35 (0.11)	0.28 (0.48e ⁻¹)	0.31 (0.35e ⁻¹)
	Loch	0.28 (0.23)	0.14e ⁻¹ (0.42e ⁻¹)	0.53e⁻¹ (0.33e ⁻¹)
<i>Length-K</i>	RAS	0.37e ⁻¹ (0.13)	-0.34e ⁻¹ (0.52e ⁻¹)	-0.69e ⁻² (0.38e ⁻¹)
	Loch	-0.36e ⁻¹ (0.26)	-0.37 (0.36e ⁻¹)	-0.33e⁻¹ (0.29e ⁻¹)

2.4.5. GxE

Quantification of GxE estimated by the genetic correlation of a trait measured in both environments (r_{gxe}) hint towards GxE effects that range from moderate-to-weak. Specifically,

the lowest r_{gxe} was seen in WBW (0.62, s.e 0.14), followed by length (0.78, s.e 0.15) and then condition factor (0.85, s.e 0.17).

2.4.6.GWAS

Based on lambda (λ) values there was weak evidence of over-dispersion (Appendix A.9). It is of note, λ was greater in RAS than loch, suggesting there may be a comparatively greater number of significant results than expected by chance. Bearing this in mind, while a greater number of SNPs were found in significant association with phenotypic traits in the RAS compared to loch environment, most of these were only significant at the chromosome level (Table 2-4). However, while no SNP met the genomic threshold for WBW, in the RAS population some were reported for length and condition factor (Figure 2-3, Figure 2-4, Table 2-4, and Appendix A.10).

Table 2-4 Number of SNPs found in significant association with phenotypic traits at the genome and chromosome level; where WBW is whole-body weight and K is condition factor.

	Genome		Chromosome	
	RAS	Loch	RAS	Loch
WBW	0	0	15	2
Length	2	0	8	8
K	6	0	13	7

The differences in the genomic distribution of significant SNPs between environments can be seen in Figure 2-2 to Figure 2-4. For WBW, significant SNPs at the chromosome level in the RAS population were found on chr 6, 8, 12, and 21. In the loch population these only fell on chr 9 (Figure 2-2). For length, in RAS significant SNPs at the chromosome level fell on chr 6, 8, 9, 10, and 12. In the loch population SNPs were also identified on chr 9 and 10, but in different regions and at a greater level of significance (Figure 2-3). Additionally, in RAS, two SNPs meeting the genomic threshold were identified on chr 12, and overlapping *pgfb* and *LOC123725452* (Figure 2-3, Appendix A. 10). In the loch population SNPs showed association to the chromosome level with condition factor on chr 13. At the same threshold, regions on chr 22 and 24 were identified in the RAS population. In this population, of note is a region identified on chromosome 22 found in significant association with condition factor (Figure 2-4), where several SNPs met the genome threshold and were found to overlap *LOC106582632*, and *ppp1r16b*, as well as neighbouring *LOC106582623* and *LOC106582673* (Figure 2-4,

Appendix A. 10). On chr 10, two further SNPs were found to overlap *irp8* and *LOC106560530*, significant at the genome level (Figure 2-4, Appendix A. 10).

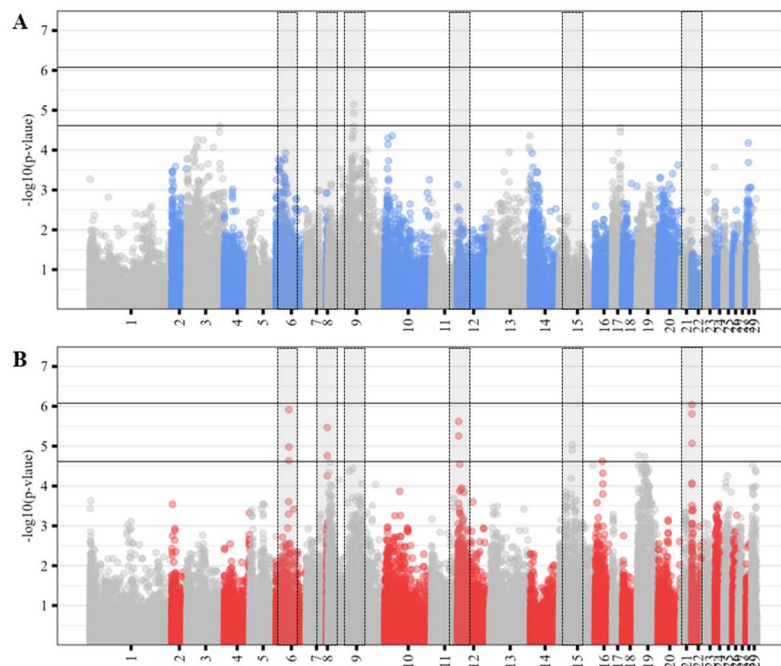


Figure 2-2 GWAS of whole-body weight (WBW) (g) in loch (blue) (A) and RAS (red) (B) environments, regions with differential SNP associations between environments highlighted in grey. Lower and upper lines indicate chromosome and genome significant levels, respectively

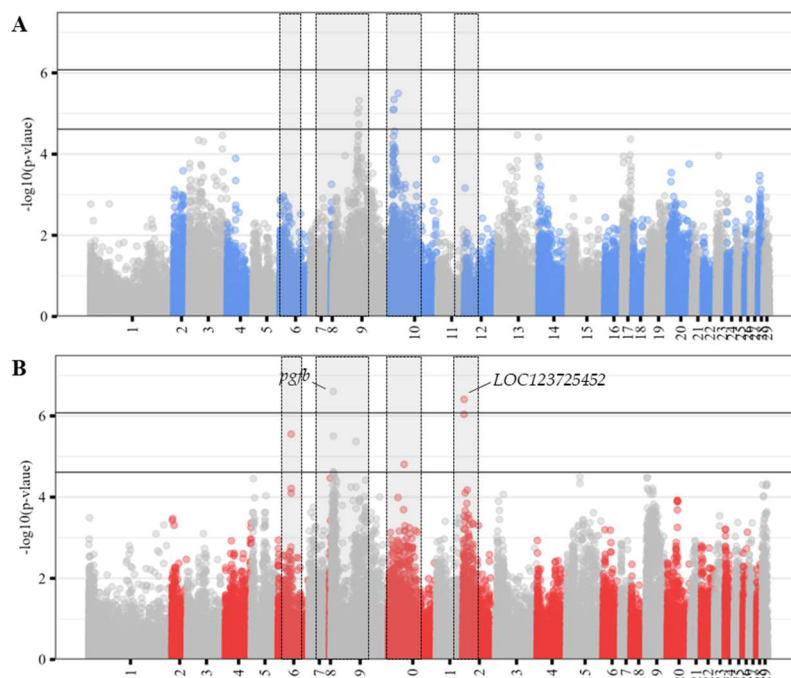


Figure 2-3 GWAS of length (cm) in loch (blue) (A) and RAS (red) (B) environments, regions with differential SNP associations between environments highlighted in grey. Lower and upper lines indicate chromosome and genome significant levels, respectively. Genes overlapped by SNPs meeting genomic threshold have been annotated.

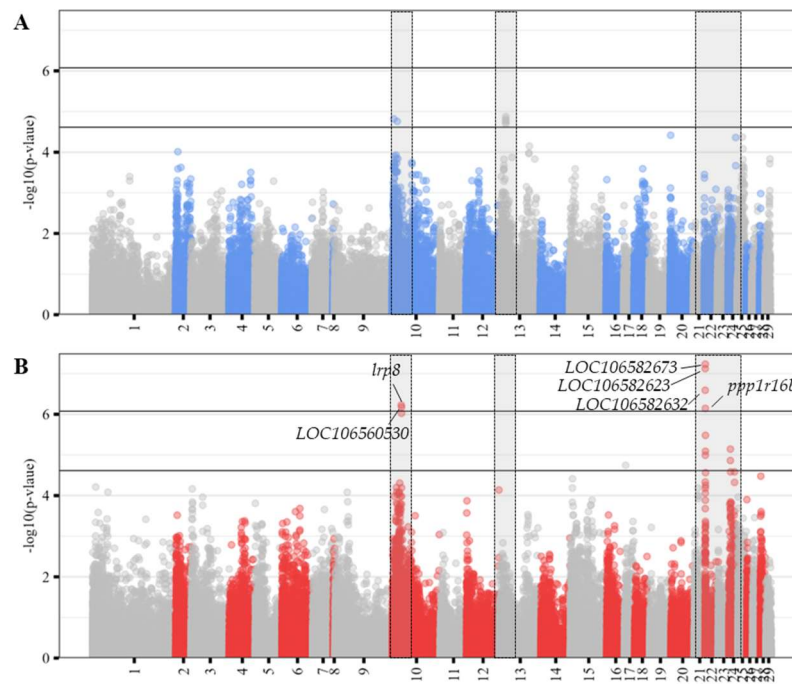


Figure 2-4 GWAS condition factor (K) in loch (blue) (A) and RAS (red) (B) environments, regions with differential SNP associations between environments highlighted in grey. Lower and upper lines indicate chromosome and genome significant levels, respectively. Genes overlapped by SNPs meeting genomic threshold have been annotated.

2.5. Discussion

2.5.1. Phenotypic data

According to our results, fish reared in the freshwater (FW) loch environment were both heavier and longer than their RAS counterparts. However, RAS fish showed on average a greater condition factor (Table 2-2). In contrast, it might have been expected that fish reared in the RAS population would have greater growth rate as they were exposed to continuous light, greater oxygen saturation, and higher water temperatures (Thrush, Duncan and Bromage, 1994; Björnsson *et al.*, 2000; Ellis *et al.*, 2016) (Figure 2-1). Consequentially, it seems that the re-use of water and high stocking densities could have affected smolts performance in RAS (Ebeling & Timmons, 2010). Specifically, high densities in RAS environments have previously been linked to decreases in weight and growth rates, as well as negatively affecting feed conversion efficiency (Liu, Liu and Sun, 2017; Wang *et al.*, 2019), in line with previous research showing the detrimental impacts of high density (Ellis *et al.*, 2002). This research has suggested that increased density negatively impacts populations via (1) increased formation of social interactions, and/or (2) deterioration of water quality (Ellis *et al.*, 2002). Based on Jobling (1995), results presented here would suggest that the poorer performance of RAS-

reared fish was due to water quality rather than social interactions (Jobling, 1995; Ellis *et al.*, 2002).

Considering this, while more research is needed to determine safe biological limits for water quality, from the values recorded in this study (Appendix A. 2), these parameters do not appear to exceed those currently seen to cause both lethal or chronic effects in salmonids when tested experimentally (Ebeling and Timmons, 2010; Kolarevic *et al.*, 2014; Becke *et al.*, 2017, 2018, 2019; Good *et al.*, 2018; Mota *et al.*, 2019). The exception is the nitrate concentration which exceeded 100mg/L, the threshold that has been experimentally tested (Davidson *et al.*, 2017). However, studies investigating the effects of high nitrate concentrations on juvenile and post-smolts have produced inconsistent results (Davidson *et al.*, 2014, 2017; Freitag *et al.*, 2015). From these potential causes, it remains unclear which underlying environmental factors could be driving the difference seen in phenotypic performance between environments, but rearing density may be a factor and pertinent to research this further. It would also be of interest to see if these differences between the two populations continued throughout the production cycle once both share the same environment post-SW transfer, as will be investigated further in Chapter 4.

Notably, in addition to FW-rearing, sex was also found to significantly impact FW performance. Overall, male fish were found to be smaller than female fish, yet within each environment, male fish were larger than female counterparts, with a sex difference within environment greater in the loch population. This contradictory pattern is suggested to result from (1) RAS-reared fish being smaller than loch-reared fish and (2) a male skew in the RAS population. Together this led to a smaller overall male population because of a dominance of small RAS fish, rather than the males themselves showing poorer growth than females.

Additionally, the nested effect of tank within environment significantly affected these results. However, within FW environments, only RAS showed a significant difference in tank performance. Between the two environments, the husbandry conditions in the loch may have been more homogenous across net-pens, due to the free movement of water between units, compared to the discrete tanks in RAS. While environmental information specific to each tank/pen was not available and further investigation into within environment variability not possible, it could be suggested that differences between individual tanks in RAS drove a

differential in phenotypic performance recorded between housed populations. This may be worth investigating further.

2.5.2. Trait variance

Estimates of trait variance seen here fall in line with previously reported values for the coefficient of variation in salmon (Gjerde and Gjedrem, 1984; Gjerde, Simianer and Refstie, 1994; Gjedrem, 2000; Fishback *et al.*, 2002; Neira *et al.*, 2004; Quinton, McMillan and Glebe, 2005; Gonzalez, Gallardo-Hidalgo and Yáñez, 2022). However, the values reported in RAS were consistently lower than the loch environment (Table 2-2). This suggests that while the difference between tank performance in RAS was significant, there was little variability around the average in each. This meant that while performance between pens was similar in the FW loch, trait variability within both was greater than across tanks in RAS. Considering this, reductions in trait variance can be achieved by husbandry manipulation, and the tighter environmental regulation offered by RAS has previously been suggested to increase consistency in family performance (Vu *et al.*, 2021). Accordingly, compared to the loch environment studied here, RAS was less variable in terms of temperature and light exposure. Other variables did, however, show greater variability (e.g. pH and oxygen saturation) (Figure 2-1).

Considering this, it is likely that the way measurements were recorded differed between environments (particularly in terms of sensor location, number or sensitivity). For example, due to the need to regulate water quantity parameters tightly in the RAS environment, monitoring of variables may have been more intensive. Unfortunately, these differences are not known, but they could have influenced variable estimates as well as variability in their means. When comparing these findings to other species, it has been observed that variance is often lower when reared in RAS compared to other husbandry environments (Sae-Lim *et al.*, 2014; Van Sang *et al.*, 2020). However, some studies have found contradictory results (Dupont-Nivet *et al.*, 2008; Dupont-Nivet *et al.*, 2010; Mas-Muñoz *et al.*, 2013; Li *et al.*, 2019).

Increasing uniformity via environmental regulation is particularly relevant in instances where phenotypic variance is largely driven by non-genetic factors. However, when it is instead genetically underpinned, uniformity can alternatively be increased through selective breeding

programs (Vu *et al.*, 2021). This relates to ideas of robustness and microenvironmental sensitivity, defined as an individual's ability to buffer against the effects of unknown biotic and abiotic disturbances within a single macroenvironment as well as developmental or endogenous disturbances (Sonesson, Ødegård and Rönnegård, 2013; Iung *et al.*, 2020). Importantly, both are increasingly becoming targets for selection (Sae-Lim *et al.*, 2016; Berghof, Poppe and Mulder, 2019). Specifically, studying microenvironmental sensitivity assumes a trait's residual variance as a proxy for uniformity before identifying its variance components. This allows estimates of breeding values and heritability for a trait's uniformity itself (Hill and Mulder, 2010; Agha *et al.*, 2018).

Despite this, studies into microenvironmental sensitivity are limited, as are practical examples of its selection (Garreau *et al.*, 2008; Iung *et al.*, 2020). However, significant estimates of genetic variance and heritability for trait uniformity have been identified in salmonids (Janhunen *et al.*, 2012; Sonesson, Ødegård and Rönnegård, 2013; Sae-Lim *et al.*, 2017; Iung *et al.*, 2020). Furthermore, in a comparison between body weight of *Litopenaeus vannamei* reared in RAS or low-density earthen ponds, greater heritability estimates were found for body weight uniformity in the RAS environment (Garcia *et al.*, 2021) (Table 1-1). This would suggest a heightened possibility of increasing uniformity through selection in RASs compared to other rearing environments. Further studies into microenvironmental sensitivity could reveal if the differences seen here in trait variance between FW environments are underpinned by differences in genetic architecture and regulation of trait uniformity in addition to possible environmental effects.

2.5.3. Within environment genetic parameters

Within environments, heritability estimates for WBW and length ranged from moderate-to-low (Navarro *et al.*, 2009a), all falling within the range reported previously for Atlantic salmon (Gjerde, Simianer and Refstie, 1994; Rye and Refstie, 1995; Quinton, McMillan and Glebe, 2005; Powell *et al.*, 2008; Yáñez *et al.*, 2014; Tsai, Hamilton, Guy, *et al.*, 2015; Tsai, Hamilton, Tinch, *et al.*, 2015; Correa *et al.*, 2018; Gonzalez, Gallardo-Hidalgo and Yáñez, 2022). While the estimated heritability for the condition factor in the loch population fell within the range reported previously (Rye & Refstie, 1995; Neira *et al.*, 2004), estimates of heritability in RAS exceeded this range. In line with this, our results suggest that the FW-rearing environment

affected heritability, where estimates across traits in RAS were over twice that of the loch environment.

This environmental difference in heritability appears a result of, firstly, a greater residual variance component in the loch environment (Table 2-2). As with the discussion of trait uniformity above, the smaller residual variance component in RAS is a hypothesised result of the husbandry control this environment permits. Conversely, the ambient environmental variability in the loch environment is suggested to have increased residual variance. Secondly (except for WBW) the RAS environment reported greater additive genetic component (Table 2-2) which will have also increased heritability estimates. Furthermore, as it accounts for differences in trait mean, CGV is often used to compare the genetic variance between environments. Values reported here reveal that the difference in V_g between environments is similarly seen in CGV where values reported were greater in RAS compared to loch environment, most clearly demonstrated in WBW. Interestingly, heterogeneous genetic variance and heritability between environments are additional indications of genotype environments interactions in addition to re-ranking (Sae-Lim *et al.*, 2014).

In contrast, correlations within environments were largely similar between the RAS and loch populations (Table 2-3). Strongly positive residual and genetic correlations were seen between WBW and length, as expected, and previously recorded by other studies in salmon (Gjerde and Gjedrem, 1984; Rye and Refstie, 1995; Tsai, Hamilton, Guy, *et al.*, 2015). Positive genetic and residual correlations were also seen between WBW and condition factor, although they were slightly greater in the RAS compared to loch environment. An interesting point of difference was seen in the genetic correlation between condition factor and length. In line with previous studies a weak negative genetic correlation was estimated between condition factor and length in the loch environment (Rye and Refstie, 1995; Fishback *et al.*, 2002; Neira *et al.*, 2004). In RAS, however, this correlation was weakly positive (Table 2-3). Given the strong positive correlation for WBW and condition factor, this indicates that selection on any one of the growth traits could act to increase the others as well, a desirable outcome. Alternatively, this could suggest slight differences in the growth patterns between populations, such that in the loch environment length is increasing at a greater rate than WBW. Other factors (e.g. feed

intake or skeletal deformity) known to affect condition could have differed between rearing environments and affected these findings.

2.5.4. Genotype-environment interactions (GxE):

Here we quantified GxE as the genetic correlation between the same trait measured in different environments (r_{gxe}). In line with suggested benchmarks (Sae-Lim, 2013; Sae-Lim *et al.*, 2016), estimates of GxE of traits measured here ranged from moderate-to-weak. Specifically, while the r_{gxe} for both condition factor and length were above 0.7, for WBW it was 0.62, suggesting a significant level of re-ranking.

The strength of GxE is often attributed to the degree of difference between environments, whereby the greater the environmental difference the stronger the GxE identified (Sae-Lim, Mulder, *et al.*, 2015; Nguyen, Hamzah and Thoa, 2017; Mengistu *et al.*, 2020). When comparing the environment of FW lochs and RAS, evidence on the RAS specific environment has previously been reviewed, highlighting stocking density, temperature, light quality and quantity, water quality, as well as environmental stability (Ebeling and Timmons, 2012; Good and Davidson, 2016; Schumann and Brinker, 2020). Here differences were seen in temperature, oxygen saturation, stocking density, and lighting conditions (Figure 1-1). Considering this, significant re-ranking was previously reported in response to temperature (McKay, Friars and Ihssen, 1984; Hebert *et al.*, 1998) and photoperiod (Stefansson, Nævdal and Hansen, 1990) in salmonids, as well as oxygen saturation in tilapia (Mengistu *et al.*, 2020). However, others have reported high r_{gxe} in response to temperature or photoperiod differences (Hanke *et al.*, 1989; Fishback *et al.*, 2002), with little investigation into GxE in relation to stocking density. Furthermore, in a cross-continental study in Rainbow trout (*O. mykiss*), degree days and photoperiod were suggested as the main environmental variables driving significant re-ranking between a nucleus population and varied production environments (Sae-Lim *et al.*, 2013, 2014).

While the analytical approach taken here considered the holistic effect of many, possibly interacting variables in each of the loch and RAS environments, it did not permit the identification of factors underpinning significant re-ranking observed for WBW, or tease these apart from one another. In the future, an alternative approach could be employed, whereby a

greater number of variables are recorded to a greater depth and shared across both environments. This would allow similar analysis to Sae-Lim *et al.* (2014), for example, whereby causal variables could be identified allowing targeted husbandry practices to reduce differences between RAS and loch environments and, in turn, the magnitude GxE.

2.5.5.GWAS

In line with previous GWAS in Atlantic salmon, few SNPs were found in significant association with phenotypic traits in either environment, which combined with moderate heritability estimates indicates a pattern of polygenic regulation (Gutierrez *et al.*, 2015; Tsai, Hamilton, Tinch, *et al.*, 2015; Yoshida *et al.*, 2017). This was most true of WBW, where no SNP was found in significant association at the genome threshold (Table 2-4, Appendix A.10). Importantly, in the RAS environment a greater number of SNPs were found in significant association with traits of interest (Table 2-4). For example, in the loch environment, no SNP met the genome threshold, across traits. This aligns with the previous findings that residual compared to genetic variance explained a greater proportion of phenotypic variance in the loch population (Table 2-2).

Our results also suggest environment-specific differences in the trait associations (Figure 2-2 to Figure 2-4). This was most evidence for condition factor where several SNPs were identified, meeting the genomic threshold. Specifically, these SNPs were found overlapping or neighbouring four genes (*LOC106582623*, *LOC106582632*, *LOC106582673*, and *ppp1r16b* (also known as TIMAP)) on chromosome 22 (Figure 2-4, Appendix A.10). While these genes have not been fully characterised, *ppp1r16b* is known to encode a 16B regulatory subunit of protein phosphatase 1 and has been functionally related to the cell cycle, regulation, and structure of endothelial cells, as well as their role in barrier protection (Celulemans and Bollen, 2004; Boratkó and Csontos, 2017). Additional SNPs meeting the genomic threshold overlapped genes (*lrp8* and *LOC106560530*) on chromosome 10 (Appendix A.10). Similarly, information on their function is limited, though *lrp8* encodes a low-density lipoprotein receptor involved in the endocytosis of ligands (Reading *et al.*, 2014), and has been related to a variety of functions in other teleosts, including vitellogenesis in *Larimichthys crocea* (Gao *et al.*, 2020), and skin coloration in *Cyprinus carpio* (Jiang *et al.*, 2022). Further investigation into these environment-specific regions may be warranted.

2.5.6. Study design

Enabled by the use of high-density SNP data, the impact of RAS-rearing during the FW development of Atlantic salmon on phenotypic and genetic parameters could be performed within commercial setting, using a commercial population. While parameters estimated from genetic analysis are often both population and environment-specific, meaning the practical applications of an experimental study can be somewhat limited, by working in a commercial setting the results presented here will have direct relevancy for the industry (Jerry *et al.*, 2006). However, this approach also has implications for the study design, which must be considered.

Firstly, prior to the split between RAS and loch environments, the population was pooled and re-allocated between tanks several times. Unfortunately, as working within a commercial setting tracking of fish (e.g. via use of PIT tags) during this period was not possible. Therefore, in the prediction models, RAS and loch were treated as a homogenous population up until their FW split and no additional environmental factors were considered. As the traits studied here reflect growth throughout the whole life cycle, they will have been affected by these previous environments (Dupont-Nnivet *et al.*, 2010; Omasaki *et al.*, 2016). The absence of these from models may therefore have affected genetic parameter estimation, especially if there were any unknown systematic differences between families, or RAS and loch populations, during this cohabitation. Of particular concern is the possible presence of common family effect that went unaccounted for. Ignoring such factors, which usually arise when families are separately reared in early development, can lead to inflation of genetic correlations between environments and false inference of weak GxE (Castillo-juárezJuárez *et al.*, 2007; Dupont-nivet *et al.*, 2010).

Secondly, sampling could not be controlled to ensure equality in family size, which was found to be highly variable. While this can impact GxE estimates, studies have suggested that REML approaches are robust to unequal family design (Sae-Lim, Komen and Kause, 2010; Sae-Lim *et al.*, 2016). Instead, the optimal sampling design to minimise bias in parameter estimates appear more dependent on family number and size (Sae-Lim, Komen and Kause, 2010). In line with this, the number of fish sampled here (1,000 per environment) has been shown to be on the low end needed to accurately quantify GxE, and while the family number (134) was sufficient, the average family size sampled here (approximately seven per environment) was

below the minimum of ten suggested by simulation studies (Sae-Lim, Komen and Kause, 2010; Lozano-Jaramillo *et al.*, 2020). Consequently, estimates presented here could have been downwardly estimated.

2.5.7.Implications for Aquaculture

Results suggest that although producing smaller fish, the RAS environment significantly reduced phenotypic variance. Increasing the homogeneity of commercial population is of interest as uniformity can (1) boost market value, (2) decrease production cost through mitigation of handling, processing, and husbandry practices, and (3) improve fish welfare (Janhunen *et al.*, 2012; Khaw *et al.*, 2016; García-Ballesteros *et al.*, 2021; Garcia *et al.*, 2021; Vu *et al.*, 2021). Differences in variance components within environments were also observed, whereby the residual variance component was larger in loch compared to RAS across traits. This translated into a greater heritability estimate in RAS, which suggests that there could be greater opportunity to apply selection in RAS.

Although the environmental parameters measured here did not reveal large differences between environments, significant re-ranking was identified for WBW. The genetic correlation of this trait between environments fell below the 0.7 threshold (Sae-Lim, 2013; Sae-Lim *et al.*, 2016). Therefore, as the production of Atlantic salmon smolts in RAS continues to increase it may be advantageous to consider incorporating RAS specific growth traits into breeding goals, including further research into RAS specific markers identified in GWAS. However, this is based only on statistical results and would incur large investment. An economic study is therefore necessary (Mulder *et al.*, 2006).

Looking forward, this work would benefit from additional information on the relative performance of RAS-reared fish throughout the full production cycle as well as more in-depth environmental data. This would help determine whether these observed growth differences and family re-ranking are maintained after their separate rearing post-SW transfer. Additionally, more in depth data could help identify possible causative factors driving observed GxE.

2.6.Conclusion

RAS-reared fish, which although smaller in terms of weight and length, had a greater condition factor and showed substantially smaller variation. A significantly smaller component of residual variance was also found in the RAS compared to the loch environment, translating into greater heritability estimates across all three traits. Importantly, significant re-ranking was identified for WBW between environments, as were differences in the genetic associations with traits.

Chapter 3- Impact of Photoperiod, Temperature, and Feeding Regime Manipulation on DNA Methylation Patterns in Atlantic Salmon, *Salmo salar*

3.1. Abstract

While the use of freshwater (FW) net-pens is still widespread in Scotland, the adoption of recirculating aquaculture systems (RAS) for the FW development of Atlantic salmon, *Salmo salar*, is increasing, in line with global trends. The environment, however, provided in RAS differs from FW lochs. There have been some indications of differential performance of RAS-reared smolts after saltwater (SW) transfer, which is hypothesised may result from developmental differences underpinned by environmentally mediated changes to the epigenome. Here, *S. salar* parr were exposed to one of six experimental treatments, comprising of combinations photoperiod, feeding regime and temperature commonly implemented in RAS. Using brain samples, reduced representation bi-sulphite sequencing (RRBS) and RNA-seq was performed to investigate methylation (DNAm) and gene expression patterns, respectively. Some indication of changes to the methylome globally was found, though this was largely absent at the scale of specific CpG sites. By contrast, transcriptomic changes due to photoperiod and feeding manipulation were identified. These changes were more apparent at high temperature where pathway enrichment of differently expressed genes (DEG) suggested changes to energy metabolism and possibly structural development. Expression changes, however, could only be weakly related to DNAm. This suggested that DNA methylation was not the mechanism by which transcriptomic changes were mediated. Together this suggests that manipulation of variables often applied in a RAS setting did not impact DNAm in a meaningful way, though findings presented here were limited and further investigation in the holistic RAS environment may be required.

3.2.Introduction

Commercial production of Atlantic salmon (*Salmo salar* L. 1758) requires several distinct husbandry environments due to its anadromous life cycle. This includes initial hatchery environments before transfer to freshwater (FW) rearing environments for smoltification (Ellis *et al.*, 2016). Smoltification is a key phase of development where FW parr undergo significant physiological, morphological and behavioural adaptations to allow successful transition to the saltwater (SW) environment (Stefansson *et al.*, 2008; McCormick, 2012).

The onset, timing, and progression of smoltification is principally controlled by the photoneuroendocrine systems (PNSE) (Migaud, Cowan, *et al.*, 2007; Migaud, Davie and Taylor, 2010). In this system, the pineal gland is directly photosensitive, and light exposure inhibits the release of melatonin, establishing daily (circadian) rhythms (Falcón *et al.*, 2010; Migaud, Davie and Taylor, 2010). Via changes to the length of nocturnal melatonin release, exposure to key photoperiod cues drives changes in the light-brain-pituitary axis, triggering a hormonal cascades that induces smolt phenotypes in parr that have reached a physiological size threshold (Stefansson *et al.*, 2008; McCormick, 2012; Nisembaum *et al.*, 2020). While unable to trigger smoltification itself, temperature additionally influences this process (McCormick *et al.*, 2002). Increased temperature is linked to faster acquisition and loss of smolt characteristics (McCormick, 2012), while decreased temperature dampens hormonal changes (McCormick, Moriyama and Björnsson, 2000; McCormick *et al.*, 2002). Other factors (e.g. water velocity and feed availability), though, can also exert an influence on salmonids FW development (Stefansson *et al.*, 2008). Commercially, successful completion of this process then allows populations to be transferred to SW farms for grow-out to harvest size.

In Scotland, while the predominant FW culture system is still net-pens in FW lochs, over recent years there has been progressive increase in the use of recirculating aquaculture systems (RAS) (Ebeling and Timmons, 2012; Clarke and Bostock, 2017). Being fully closed land-based systems, where water is recycled and its quality constantly maintained through various filtration mechanisms (Ebeling and Timmons, 2012), RAS offers various advantages over open water net-pen systems (Ebeling and Timmons, 2012; Kolarevic *et al.*, 2014; Mota *et al.*, 2020). Additionally (as explained in the previous chapters), there are differences in environmental parameters (e.g. stocking density, temperature, photoperiod etc.) in RAS

compared to traditional culture systems, with the daily variability in these factors also differing (Ebeling and Timmons, 2012; Good and Davidson, 2016; Clarke and Bostock, 2017; Schumann and Brinker, 2020).

Epigenetics, understood as chromosome-bound changes to gene expression which are either mitotically or meiotically heritable, represents a layer of gene regulation above that of the DNA sequence itself (Waddington, 1942; Deans and Maggert, 2015; Eirin-Lopez and Putnam, 2019). Various mechanisms of epigenetic regulation of gene expression have been identified (discussed in detail in General Introduction, Chapter 1.4), with DNA methylation (DNAm) the most widely understood and studied (Clark and Rager, 2020). Although the majority of the vertebrate genome is unmethylated, methylation principally occurs at CpG sites, where the majority cytosines are methylated (Long *et al.*, 2016; Ambrosi, Manzo and Baubec, 2017; Eirin-Lopez and Putnam, 2019). This process is catalytically performed via the action of DNA methyltransferases (DNMTs) (Best *et al.*, 2018). Methylation is not, however, fixed and can be lost actively via the action of TET enzymes, or passively through cellular division (Best *et al.*, 2018) (for details see General Introduction, Chapter 1.4.1.1).

Importantly, DNAm can be shaped by environmental exposure, such as specific variables (Marandel *et al.*, 2016; Burgerhout *et al.*, 2017; Uren Webster *et al.*, 2018) or holistic environments (Le Luyer *et al.*, 2017; Rodriguez Barreto *et al.*, 2019; Wellband *et al.*, 2021) (detailed in General Introduction, Chapter 1.4.2). Critically, DNAm changes can have phenotypic consequences due to a downstream effect on gene expression. Broadly speaking, this occurs via methylations effect on transcription factors and their affinity for binding sites (transcription factor binding sites, TFBS). Canonically, this is a repressive process, though over recent years it has become understood to be context and location specific (Feil and Fraga, 2012; Zhu, Wang and Qian, 2016; Eirin-Lopez and Putnam, 2019). Harnessing these effects of DNAm are now of interest for aquaculture. For example, broodstock, eggs or larvae can be exposed to specific environmental stimuli that programs the epigenome for future exposure (so called 'priming'). Conversely, across husbandry environments, initial conditions may set mal-adaptive epigenetic signatures that inhibit later life performance (Araki *et al.*, 2008; Taudt, Colomé-Tatché and Johannes, 2016; Gavary *et al.*, 2019; Roy *et al.*, 2021).

This study suggests the environmental variables often manipulated in RAS may alter DNA methylation patterns in a novel way, potentially affecting performance. To investigate the possibility of this, Atlantic salmon parr were exposed to combinations of feeding, photoperiod and temperature regimes, with the aim of identifying and characterising (1) the brain methylome, (2) differently expressed genes, and (3) differently methylated cytosine between treatment groups, as well as (4) investigating if any identified changes in gene expression and cytosine methylation can be linked.

3.3. Materials and Methods

3.3.1. Ethics

Animal handling and collection in this study was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 Amended Regulations (SI 2012/3039) and in accordance with EU regulation (EC Directive 86/609/EEC). All experimental work was approved by the University of Stirling Ethics Committee (Animal Welfare and Ethics Review Board; (Epigenetic regulation in Salmon: AWERB 2021 1977 1742).

3.3.2. Experimental design

Experimental fish for this study originated from the stock housed at the Niall Bromage Freshwater Research Unit (NBFRU; Buckieburn, University of Stirling). Approximately 2,500 fish of 15g were transferred to temperate aquarium, University of Stirling. The population was split evenly across 12 tanks. Each tank was 85cm in diameter, 55cm in height, with a usable volume of approximately 312 litres. They were held at 12° C and exposed to continuous light and feed for 12 days (Appendix B. 8). Fish were starved overnight before being pooled into three tanks and randomly allocated between 18 tanks in batches of 20. Numbers were finally evened out between tanks, approximating 125 fish per tank.

Fish were then exposed to one of six treatments (Table 3-1), each run in triplicate. Treatments were a combination of photoperiod, feeding, and temperature regimes. Of note, control conditions were the same as those of the baseline (Table 3-1). Each triplicate treatment was housed next to each other in adjacent tanks, with temperature regimes split between the two available systems, where system A was the high temperature, and system B the low

temperature group (Appendix B. 8 and Table 3-1). Experimental treatments lasted for approximately 800 degree days (dd), calculated from the point fish were allocated between tanks for experimental treatment. Specifically, dd were calculated as $T^{\circ}\text{C} \times \text{number of days}$, where $T^{\circ}\text{C}$ is degree Celsius. $T^{\circ}\text{C}$ was 11°C and 15.5°C for low and high temperature treatments, respectively.

Table 3-1 Treatment groups, comprising of continuous light: LL, or 18:6 day night cycles: LND, continuous feed: CF, or feeding in the perceived day: DF, and temperature of 11.5°C : LT or 15.5°C : HT. Control conditions are indicated by * (constant light and constant feed).

Photoperiod	Feeding regime	Temperature
LL*	CF*	LT*
LL	DF	LT
LND	DF	LT
LL*	CF*	HT*
LL	DF	HT
LND	DF	HT

Environmental data over experimental period was automatically recorded daily by the facilities system. Measurements of maximum temperature ($^{\circ}\text{C}$), nitrite (mg/l), chloride (mg/l), oxygen saturation (%) and total available ammonia (TAN, average mg/l) were taken. For each, the average and standard deviation (s.d) were calculated over the whole experimental period (~800dd for each system).

3.3.2.1. Sample collection

The population was sampled twice. First, baseline samples were taken before onset of experimental treatments. Two fish from three tanks (Appendix B. 8) were selected at random. Secondly, the population was sampled after ~800dd of exposure to experimental treatments. Six fish per treatment (two per tank) were sampled (Appendix B. 8). In both cases, tissue samples from fish were collected following lethal dose of anaesthesia and following UK schedule 1 protocol. Brains were removed intact and whole as possible before removal of the pituitary gland to minimise background noise in analysis. Samples were snap-frozen using liquid nitrogen in cryovials [Thermo scientific limited] and stored at -70°C until analysis. Additionally, measurements of whole-body weight (WBW) and length (tip of head, snout, to the deepest point of the fork in the caudal fin) were taken on all sampled fish.

3.3.2.2. Feed intake assessment

To assess if feeding performance was affected by environmental manipulation, feed intake assessment was performed for two weeks after all sampling was performed. In triplicate for each system, ~10g of dry feed was full soaked in the system water for approximately 6 hours. The average ratio of wet to dry weight ratio was calculated.

At the end of the experimental period feeding times were adjusted to pause feeding from 8:30 to 10:30 and 15:00 to 17:00 each day during which waste from each tank was siphoned over a sieve. Waste was manually washed to remove faeces, lightly blotted with blue roll to remove excess water, and wet weight of uneaten feed recorded (WW_{waste}). This value was converted to dry weight (DW_{waste}) using the estimated ratios. Dry weights of feed provided per tank (DW_{feed}) were recorded automatically by the Arvo-tec system. DW_{waste} and DW_{feed} were converted to grams. Morning and afternoon measurements of DW_{waste} and DW_{feed} were combined per day and adjusted to a 24-hour period running from 4pm to 4pm. Number of fish per tank was recorded per day (n_{tank}), accounting for any mortalities. From these values, the daily available feed per fish (mg/fish) was calculated as $(DW_{feed}/n_{tank}) * 1000$, and feed intake (FI) per fish (mg/fish) as $(DW_{feed} - DW_{waste}/n_{tank}) * 1000$. Average and standard error (s.e) were calculated across triplicate tanks per treatment.

Over the assessment period, total feed consumed (FC_{kg}) was calculated as the sum of daily FI measurements and converted to kg. Fish weight (g) estimates, per tank, automatically recorded by the Arvo-tec system, were taken both at the start (W_{start}) and end (W_{final}) of this period. From this, the start biomass (kg) (Bio_{start}) was calculated as $(W_{start} * n_{start})/1000$ where n_{start} is the initial number of fish per tank, the end biomass (kg) (Bio_{final}) was calculated as $(W_{final} * n_{final})/1000$ where n_{final} is the final number of fish per tank, and mortality biomass (kg) (Bio_{mort}) was calculated as $((n_{start} - n_{final}) * Average(W_{start}, W_{final}))/1000$. From this the following was calculated: average FI ($FI_{average}$) per fish per day (g/fish/day) was calculated as $((FC_{kg}/days)/n_{final}) * 1,000$, FI as a percentage of weight ($FI_{\%BW}/day$) as $(FI_{average}/Average(W_{start}, W_{final})) * 100$, and feed conversion ratio (FCR) as $FC_{kg}/((Bio_{final} + Bio_{morts}) - Bio_{start})$. Values were calculated per tank, with the average and standard error (s.e) calculated across triplicate tanks for each treatment.

3.3.3.RNA and DNA extraction, library preparation and sequencing

Brain samples were allowed to thaw on ice and placed into 1ml screw cap tubes. 650µl of TEx1 and 2.7mm glass beads were added. Samples were homogenised in bead beater [BioSpec products, Bartlesville, OK, USA] at full speed for 35 seconds.

3.3.3.1.DNA extraction

DNA was extracted following a SSTNE/Salt precipitation protocol. After homogenisation, samples were allowed to slightly settle, 200µl of the homogenized samples were then pipetted into 20µl of 10% SDS, 200µl of SSNTE buffer and 5 µl Proteinase K (10mg/ml). Samples were mixed by inversion and placed into incubator overnight at 55°C. Samples were then heated to 70°C in Dri-block (heat-block) [Techne] and allowed to cool to room temperature before 1µl RNase A (2mg/ml) was added. Samples were incubated at 37°C for 1 hour. 70% of the total volume of (5 molar) sodium chloride (NaCl) was added to samples and were left to stand on ice for 10 minutes. Samples were centrifuged at 21,000 x g at room temperature for 10 minutes [Sigma, USA]. The supernatant was removed and added to an equal volume of isopropanol [Fluka, Switzerland] and mixed by inversion. Samples were centrifuged as before. All traces of isopropanol were then removed, leaving the formed pellet undisturbed. 1ml of 70% ethanol was added and placed into rotator at room temperature for at least an hour. All traces of the ethanol were removed before this last step was repeated. DNA samples were re-suspended in 50µl DNA re-hydration solution.

3.3.3.2.RRBS library preparation and sequencing

The concentration and quality of DNA samples were assessed using Nanodrop [labtech, East Sussex, UK], dsDNA broad spectrum Qubit [Invitrogen, Waltham, MA, USA], and gel electrophoresis. Samples were diluted to approximately 100µg/µl. Sample concentration was checked using dsDNA high sensitivity Qubit [Invitrogen, Waltham, MA, USA] and diluted to approximately 20µl/µl. With the assistance of Dr. Alejandro Gutierrez and Dr. Callum Howard, reduced representation bi-sulphite sequencing (RRBS) library preparation was performed using Diagenode premium RRBS kit x96 [Diagenode, Liege, Belgium] (see full protocol at Diagenode (2021)). Following manufacturers' instructions bisulfited conversion was performed, followed by amplification and pooling. In brief, this method transformed

unmethylated cytosines first to thymine and then uracil base, while methylated cytosines remained unaffected allowing their detection. Importantly, DNA samples were first digested with a restriction enzyme (MspI) which targeted 5' -CCGG- 3' sequences. This means that after size selection of digested DNA, samples were enriched for CpG rich regions which are likely to be of biological significance (i.e. promoters) (Kurdyukov and Bullock, 2016; Diagenode, 2021). Conversion was checked using bioanalyzer 2100 [Aligent, USA]. Samples were sent to Genewiz-Azenta [Leipzig, Germany] for sequencing on NovoSeq 2x150 platform.

3.3.3.3.RNA extraction and sequencing

From homogenized brain samples, 200µl for RNA extraction was pipetted into 1ml TRI Reagent [Ambion, Sigma, ABgene, Invitrogen], mixed by inversion, and left to sit on ice for approximately 30 min. Samples were stored at -70° C until extraction.

Samples were removed from -70° C freezer and allowed to thaw on ice under fume hood. 100µl BCP (1-bromo-3-chloropropane) [Sigma, USA] was added. Samples were shaken vigorously and allowed to stand for 15 min. Samples were centrifuged at 20,000 x g and 4° C for 15 min [Sigma, USA]. The top layer was removed (~700µl) and placed into fresh tube. ~350µl (half of the volume of the removed top layer) of RNA precipitation solution and 100% isopropanol [Fluka, Switzerland] were added. Samples were mixed by inversion and incubated at room temperature for 10min. Samples were centrifuged at 20,000 x g and 4°C for 10 min. After centrifugation, 1ml 75% ethanol was added and the samples were left for 15 min at room temperature. Samples were briefly centrifuged again, and all traces of ethanol removed. The remaining RNA pellet was allowed to air dry before re-suspension in in 70µl milliQ water.

The concentration of samples was measured using Nanodrop [labtech, UK] and diluted to ~100ng/µl in a total volume of 35µl. Samples were then sent to Novogene [Cambridgeshire, UK] on dry ice for RNA sequencing. Specifically, RNA samples underwent quality control (QC) and sequencing on Novaseq 6000 paired end 150 platform.

3.3.4.Data preparation

3.3.4.1.RRBS data processing and methylation calling

RRBS data pipeline largely follows those presented in Mukiibi *et al.* (2022) and Chen *et al.* (2018).

Using fastp (v.0.23.1) (Chen, 2023) sequenced reads were filtered to remove short reads (length <100bp), reads with low quality (<20 phred value) and low complexity (<20%). Automatic removal of adaptors was enabled with fastp function `--detect_adapter_for_pe` and specific adaptor sequences were filtered out using the function `--adapter_fasta`. The Atlantic salmon genome (Ssal_v3.1) was bisulphite converted using the Bismark tool `bismark_genome_preperation` (Bismark v.0.22.3) (Krueger and Andrews, 2011) before filtered reads were aligned to the converted genome (Bismark internally uses Bowtie 2). Methylation state at each site was then extracted using Bismark tool `bismark_methylation_extractor`.

Using Bioconductor R package edgeR (v3.24.2), CpG sites were filtered (Chen *et al.*, 2018). The sum of methylated and unmethylated reads were calculated and taken as the coverage for that site. Across all samples, CpG sites were removed if they had low coverage (<8 reads per sample total) or located on unplaced scaffolds without annotated genes associated to them. These filtered CpGs were the background set retained for all further analysis.

3.3.4.2.CpG site annotation

The proportion of methylated reads was calculated as $Me/(Me + UnMe)$ (where Me is methylated and UnMe unmethylated reads) for the background CpGs. CpGs were annotated to DNA strands using Bismark tool, `cytosine.2.coverage`, before functional annotated to the salmon genome (Ssal_v3.1, RefSeq GCF_905237065.1) using the `annotatePeaks.pl` tool from the HOMER software package (<http://homer.ucsd.edu/homer/ngs/annotation.html>). Under this, CpG sites were assigned to the nearest annotated gene, with the distance in base pairs (bp) recorded, and classified under different genomic features. These were as follows: promoter-TSS: putative proximal promoters/transcription start site (TSS) regions (located -1kbp to +100 bp around the TSS), exons, introns, TTS (transcription termination site, defined as regions -100 bp to +1kbp around the TTS), or in intergenic regions. From this information, the 1st exon

and 1st intron were distinguished, with remaining CpG in each category classed as other-exons and other-introns.

For each CpG site, average methylation proportion and its standard deviation (s.d) was calculated for the baseline, control, and each treatment group. Where these were 1 or 0, CpGs were classed as always-methylated or always-unmethylated, respectively. CpG sites with values falling between 1 and 0 were classed as having variable methylation.

3.3.4.3.RNA-seq data processing and annotation

Libraries were cleaned using fastp (v 0.23.1). Short reads (<150 bp) (`--length_required 150`), those with low quality (< 20 phred value) (`-q 20`), with low complexity (<20%, defined as the percentage of bases different from their next base) (`-Y 20`) or containing more than one 'n' base (`-n 1`) were removed. Additionally, automatic removal of adaptors was enabled with `--detect_adapter_for_pe` and specific adaptor FASTA sequences filtered out (`--adapter_fasta`) (Chen, 2023). Reference genome (Ssal_v3.1, RefSeq GCF_905237065.1) was indexed using Hisat2 to facilitate faster downstream steps. Paired end reads were aligned to this reference genome using Hisat2, outputting the file in SAM format (Kim, Langmead and Salzberg, 2015). Aligned reads were converted from SAM to BAM file using SAMtools (Danecek *et al.*, 2021). To estimate expression of transcripts, abundance was estimated by Stringtie (Pertea *et al.*, 2015, 2016). Resultant count data was converted to a matrix. Data was normalized as fragments per kilobase million (FPKM).

3.3.5.Data analysis

Treatment groups (Table 3-1) within high and low temperature groups were analysed separately due to observed difference in growth performance (Appendix B. 1 and B. 3). Unless otherwise stipulated data was visualized using *ggplot* (v3.4.2) in R (v4.3).

3.3.5.1. tSNE on global transcription and methylation

Excluding baseline measurements, how samples clustered was investigated via t-distributed stochastic neighbour embedding (tSNE). This was performed in R (v4.3) using *Rtsne* (v0.16) for a maximum of 5,000 iterations, without checking for duplicates, perplexity of 4, and with

a learning rate of 20,000. This was performed for FPKM gene expression as well as the proportion of methylated reads at each CpG site.

3.3.5.2. Identification of differentially methylated cytosines (DMC) and differentially expressed genes (DEG)

Identification of DMC was performed using a Bioconductor R package edgeR (v3.24.2) using a negative binomial generalized linear model (Chen *et al.*, 2018). Firstly, to account for changes over the 800dd not due to environmental manipulation, DMC which had changed over time (DMC_{time}) were identified by comparing baseline samples to those exposed to the control conditions (LL.CF) over the experimental period (Table 3-1). Specifically, the control group at low temperature group was LL.CF.LT, and high temperature it was LL.CF.HT. Please note, this means a ‘true’ baseline was not present for the high temperature group, and DMC_{time} may have been influenced by change from low temperature at baseline to high temperature at 800dd.

Secondly, a series of pairwise comparisons were made on samples from 800dd between those under ‘control conditions’ and those exposed to ‘environmental manipulation’ (DMC_{800dd}) (Table 3-2). In each, treatment groups exposed to a ‘reference’ condition were compared to treatment groups exposed to ‘experimental’ condition (Table 3-2). This was performed for photoperiod (LL vs LND), feeding regime (CF vs DF), as well as photoperiod and feeding regime combinations (LL.CF vs LL.DF and LL.CF vs LND.DF). DMC_{800dd} unique to 800dd and not also differentially methylated through time (i.e. DMC_{time}) were then identified. These final lists of DMC_{800dd} were then sorted based on their P-value (smallest first) and hierarchical clustering of the top 100 were visualised with heatmaps using the *heatmap.2()* in R.

Table 3-2 Pairwise comparisons to identify DMC_{800dd}, the samples included under each experimental condition has been demonstrated.

	Control conditions		Environmental manipulation	
	Reference	Treatment groups	Experimental	Treatment groups
<i>Photoperiod</i>	LL	LL.CF; LL.DF	LND	LND.DF
<i>Feeding regime</i>	CF	LL.CF	DF	LL.DF; LND.DF
<i>Photoperiod x</i>	LL.CF	LL.CF	LL.DF	LL.DF
<i>Feeding regime</i>	LL.CF	LL.CF	LND.DF	LND.DF

Both DMC_{800dd} and DMC_{time} were filtered using a likelihood ratio test at 0.01% significance level ($p\text{-value} < 0.001$) and false discovery rate (FDR) of < 0.01 . Putative DMC_{800dd} and DMC_{time} were then identified at $p\text{-value} < 0.001$ only. For DMC_{time} where the average proportion methylation of a DMC was greater at the baseline compared to control conditions at 800dd it was considered hypo-methylated. Conversely, when the average proportion of methylation was lower in the baseline it was considered hyper-methylated. Similarly, for DMC_{800dd} , where average methylation of samples under control conditions was greater than those under environmental manipulation, it was considered hypo-methylated. When the average methylation of samples under control conditions was less than those under environmental manipulation, it was considered hyper-methylated. Genomic annotation of DMC_{800dd} between treatment groups was compared to the background set of CpG using a Chi-square test for Goodness of fit (significant at 5%). Genomic annotation of hypo- and hyper-methylated DMC_{800dd} within treatment groups was compared using Chi-square test for association (significant at 5%). Genes annotated to DMC_{800dd} and DMC_{time} were then identified (DMC_{gene}).

Identification of differentially expressed genes (DEG) was performed in R package DESeq2 (v1.40.2) and performing the same series of pairwise comparisons as in differential methylation analysis. DEG were filtered at $p < 0.001$ and $-\log_{2}$ fold change > 1 thresholds. Results were visualized in volcano plots using *plotMA()* function from DESeq2. DEGs with a positive $\log_2$ fold change were considered up-regulated both through time (DEG_{time}) and in sample exposed to environmental manipulation (DEG_{800dd}). Those with a negative $\log_2$ fold change were considered down-regulated.

3.3.5.3. Functional enrichment (KEGG and GO)

For LND, DF, LL.DF and LND.DF treatment groups, at both temperatures, the set of unique and overlapping DMC_{800dd} , DMC_{gene} and DEG_{800dd} were identified. This was visualised in R (v4.2) using function *ggvenn* (v0.1.10). The 'meaning' of each segment can be seen in Figure 3-1. Where enough DEG or DMC_{gene} were identified, gene sets were tested for KEGG enrichment (ShinyGO v0.80, Ge, Jung and Yao, (2020)), available at: <http://bioinformatics.sdstate.edu/go/>) against Atlantic salmon genes (Ssal_v3.1), with FDR < 0.05 , minimum pathway size of 2 and maximum of 5,000 (default settings). Where this was not the case, GO annotations for individual genes were recorded from Ensembl

(<https://www.ensembl.org/index.html>). For DEG, a background set of genes was entered as all those present in the RNA-seq count matrix. For DMC_{gene}, a background set of genes was entered as genes that overlapped with filtered CpG sites.

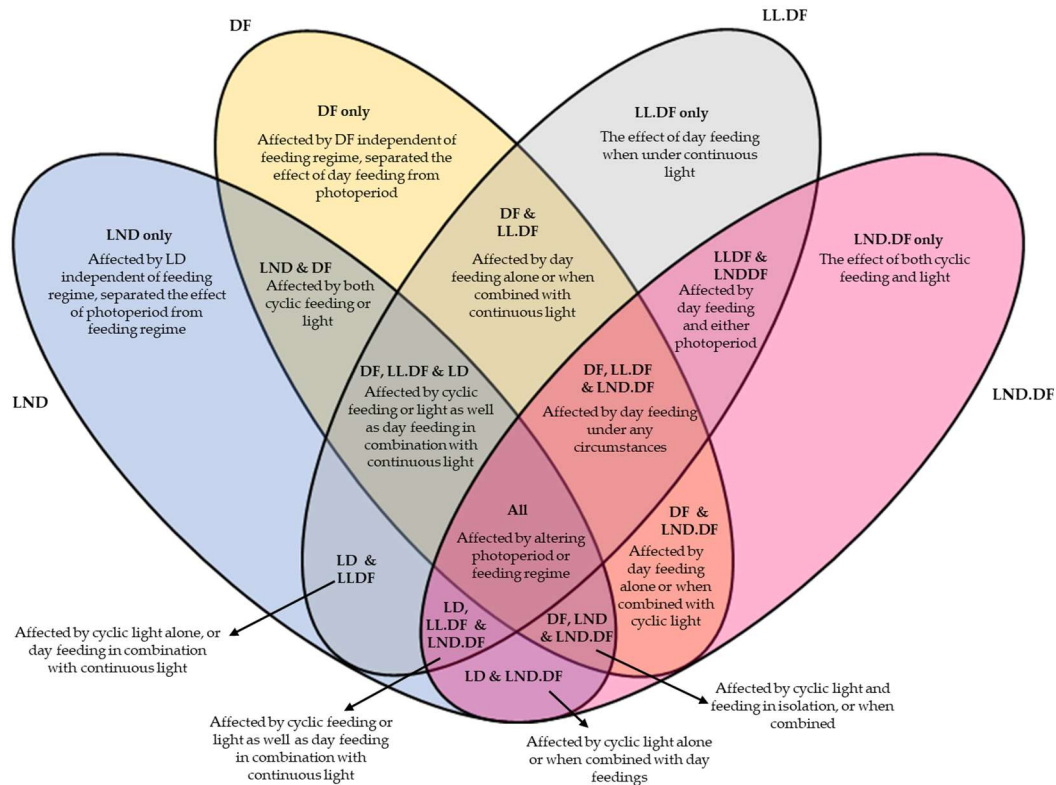


Figure 3-1 The meaning or interpretation of unique and overlapping sets of genes overlapped by DMC_{800dd}, DMC_{genes} or DEG_{800dd}

3.3.5.4. Integration of methylation and transcription data

The (Pearsons) correlation was calculated between its methylation proportion and normalized (FPKM) expression of the overlapping gene for all CpG sites. Sites where a correlation could not be calculate was classed as non-calculable. Those were this could be done were taken as significant at $p < 0.05$, or else were non-significant. This was further split by the nature of the correlation (i.e. positive or negative). The proportion of CpG sites annotated to different genomic features in significant or non-significant associations were investigated. For each genomic feature, the overarching correlation between the average methylation proportion and FPKM was calculated. This was based on the average taken across all samples, as well as the baseline and treatment groups separately. Statistical significance was taken at 5%. Lastly, the overlap between DMC_{800dd} and DEG_{800dd} for each treatment group was investigated.

3.4.Results

3.4.1.Environmental description

System A represented the high temperature treatment, and the average maximum temperature measured over the 800dd experimental period was 15.46°C (Table 3-3). In comparison, system B, the low temperature treatment, had an average of 11.90°C. While average oxygen saturation and total available ammonia (TAN) were comparable between systems, system A showed greater TAN peaks and system B showed a drop in oxygen saturation over the experimental period (Appendix B.8-B.9). Average maximum nitrite and chloride were greater in system A (Table 3-3).

Table 3-3 Mean and standard deviation (s.d) for environmental variables across the experimental period (~800dd). System A was used for the high temperature and system B the low temperature groups. TAN is total available ammonia.

	System A	System B
<i>Max Temperature (°C) (s.d)</i>	15.46 (0.32)	11.90 (0.46)
<i>Max Nitrite (mg/l) (s.d)</i>	0.60 (0.15)	0.80 (0.10)
<i>Max Chloride (mg/l) (s.d)</i>	215.10 (22.06)	219.05 (10.11)
<i>O₂ saturation (%) (s.d)</i>	92.00 (0.00)	91.88 (0.31)
<i>TAN (average mg/l) (s.d)</i>	0.14 (0.04)	0.12 (0.03)

3.4.2.Sample exclusion

Five samples were removed from all further analysis. One was identified as an outlier based on the recorded length (Appendix B. 1). Three were removed due to above average duplication rates in RNA-seq data (specifically 52.6%, 63.3% and 64.7%) (Appendix B. 6). One further was removed due to low number of sequences reads obtained from its RRBS library (3,444,930 reads corresponding to 134,988 CpGs post QC) (Appendix B. 2). This left only four samples for LL.DF.LT and LL.CF.HT, and five for LND.DF.HT. Of note, for LL.CF.HT, both removed samples originated from the same tank, meaning one replicate tank was lost.

3.4.3.DNA methylation

3.4.3.1.RRBS libraries

Full sample information can be seen in Appendix B. 2. On average 56,141,832.32 (s.e 2,783,896.53) reads were obtained before filtering, though this varied largely. Post filtering,

47.66% reads were removed on average. Of sequence pairs analysed, on average, 52.48% mapped uniquely to *Salmo salar* genome assembly, corresponding to 283,066,477.8 (s.e 17,508,988.56) cytosines analysed, 17.96% of which were at CpGs. Of all cytosines, the minority were methylated (13.51%). However, the vast majority of these methylated cytosines fell within the context of CpGs (96.09%). Equally, the majority of CpGs themselves were methylated (71.55%).

3.4.3.2. Background CpGs and patterns of genomic methylation

A total of 42,359 CpG sites met the filtering criteria. The majority were annotated to intergenic and intronic regions (Figure 3-2). Specifically, 19,131 were intergenic, 12,572 intronic, 5,543 annotated to the 1st intron, 1,606 located in promoters-TSS, 1,589 exonic, 1,081 annotated to the 1st exon, and lastly 837 were in TTS. A total of 8,322 protein coding genes were annotated to background CpGs. The distribution of CpG sites relative to TSS showed a bell curve, and was highly concentrated around the TSS, with 50% of all data falling between 15,689 bp upstream and 17,172 bp downstream of the TSS (Appendix B. 13). A similar distribution was seen when split by genomic annotations, but with different maximal distances from the TSS. This was at the exception of 1st exon and promoter-TSS where in the former CpGs were concentrated downstream of TSS, while the latter showed a bi-modal distribution with peaks at ~ 750bp upstream of TSS and at TSS itself (Appendix B. 13). Furthermore, across features, the majority of CpGs were annotated to a single gene (Appendix B. 12), with a secondary cluster where ~5-10 CpGs were annotated to the same gene. Notably, TTS showed a tertiary cluster with ~15 CpGs per gene.

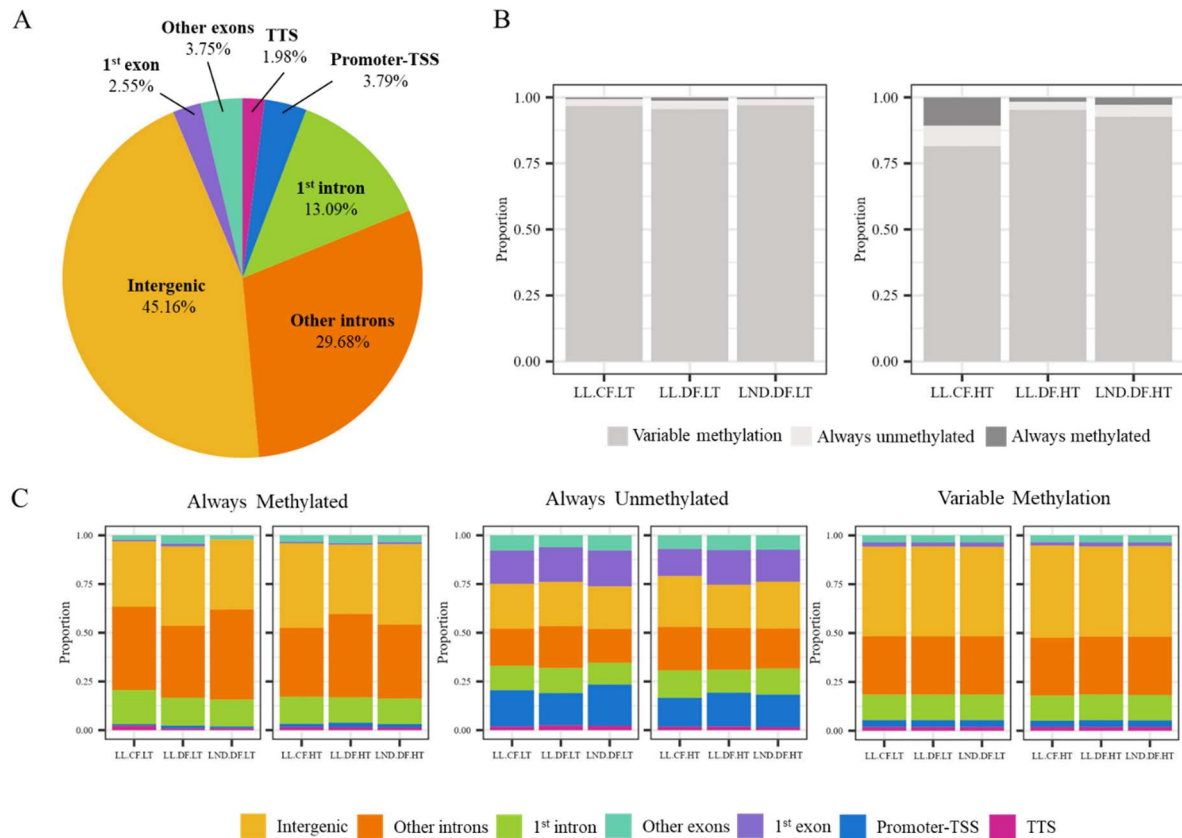


Figure 3-2 A) Pie chart demonstrating the percentage of CpG sites annotated to different genomic features, B) the proportion of CpGs under each experimental treatment that showed variable methylation or found as always-methylated or always-unmethylated, and C) the genomic annotation of CpG of different methylation status per treatment group. LT is low temperature, HT is high temperature, CF is continuous feeding, DF is feeding in the perceived day, LL is continuous light and LND is light and dark cycles.

Most CpG sites showed variable methylation, with this portion greater for fish held at low temperature compared to those in high temperature (Figure 3-2). At low temperature, there were proportionally more always-unmethylated CpGs compared to always-methylated sites. This was also true at high temperature at the exception of LL.CF.HT group. The annotation of CpG with variable methylation or consistent methylation were similar, and aligned with their proportional make up across all CpG sites. Unmethylated cytosines showed a greater proportion of CpG falling within the 1st exon or promoters-TSS, and fewer within intergenic and other-introns. This pattern was largely similar between treatments (Figure 3-2).

The 1st exon and promoter-TSS showed lower average methylation compared to other genomic features, though all had high estimates of error (Figure 3-3). Across genomic features, the distribution of methylation proportion was bi-modal showing peaks at approximately 0 and 1, but differences were seen between features (Figure 3-3). Intergenic, intronic (other-introns and 1st intron) CpGs, as well as those annotated to TTS, showed a greater peak at 1

than 0 methylation proportion. Other-exons showed near equal peaks at 0 and 1. In contrast, CpGs annotated to either promoter-TTS or the 1st exon showed a greater peak at 0 than 1. Between temperatures, bi-modal distribution was more extreme in the high temperature group, with peaks closer to 0 and 1. Between treatments, slight differences were only seen in LL.CF.LT and LL.CF.HT groups where the former showed a shift to a lower proportion whereas the latter showed a shift to higher proportion of methylation when compared to the other treatments

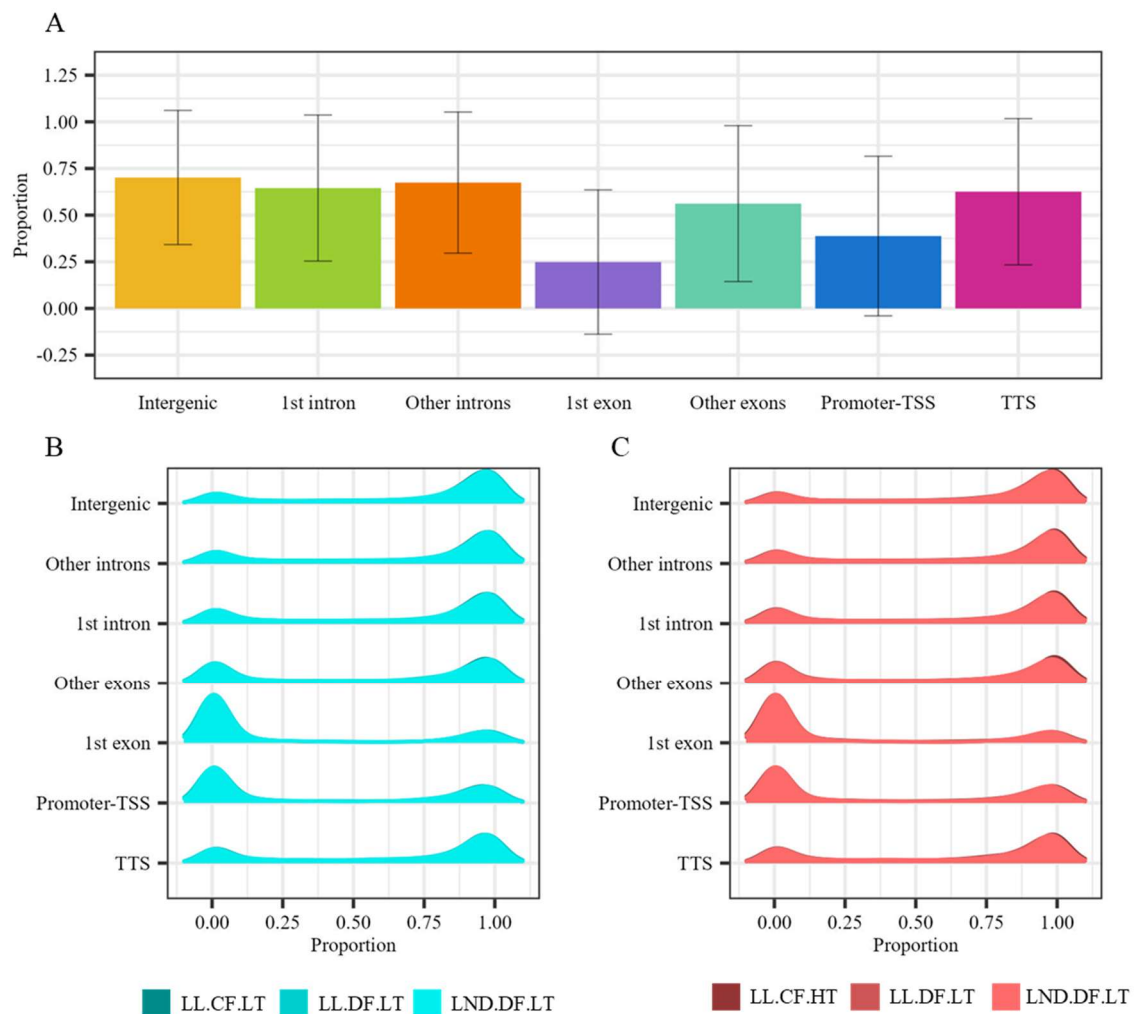


Figure 3-3 A) Average and standard deviation (s.d) of methylation proportion per genomic features, calculated across all samples. (B) and (C) show the distribution of CpG methylation proportion for the low and high temperature groups, respectively, split by genomic feature and experimental treatment. In this, LT is low temperature, HT is high temperature, CF is continuous feeding, DF is feeding in the perceived day, LL is continuous light and LND is light and dark cycles.

3.4.4.. Photoperiod and feeding regime induced methylation changes

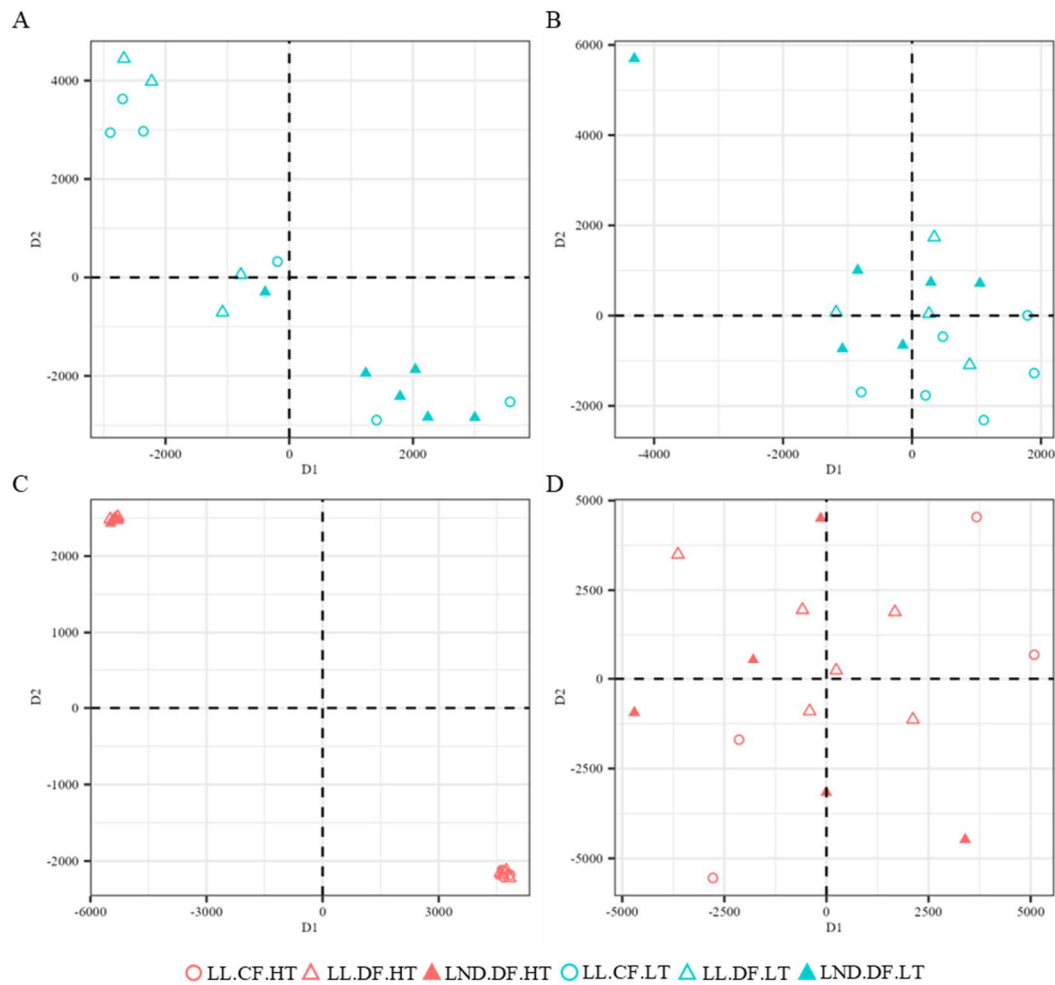


Figure 3-4 Visualisation of the first two dimensions of tSNE analysis for FPKM gene expression (A and C) and the proportion of methylated reads at each CpG site (B and D). This was performed for both the low (A and B) (blue) and high (C and D) (red) groups. Circles are those exposed to continuous feeding (CF), triangles to feeding in the perceived day (DF), filled in shapes exposed to day night cycles (LND) and empty to continuous light (LL).

Hierarchical clustering of the top DMC_{800dd} in terms of significance (i.e. lowest p-value) reflected treatment conditions, except for the photoperiod at low temperature where one sample showed anomalous clustering (Appendix B. 14). In contrast, dimension reduction revealed little clustering by treatment based on methylation proportion across all CpG sites (Figure 3-4). Similarly, no or very few CpG sites were identified as differently methylated (Table 3-4). However, a number of putative DMC were identified, where, broadly speaking, comparable numbers were recorded between treatments (Table 3-5).

Chapter 3 - Transcriptional and epigenetic responses to environmental manipulation

Table 3-4 Annotation of DMC meeting both FDR and P-value thresholds at both high and low temperatures. In this, Chr is chromosome, logFC is log-fold change, FDR the false discovery rate, Entrez.ID is the gene ID from Ensembl, LT is low temperature, HT is high temperature, DF is feeding in the perceived day, CF is continuous feeding, LND is light dark photoperiod, and LL is continuous light.

		Chr	Locus	logFC	p-value	FDR	Entrez ID	Genomic feature
LT	Time	-	-	-	-	-	-	-
	DF	-	-	-	-	-	-	-
	LND	-	-	-	-	-	-	-
	LL.DF	1	116074749	5.3029032	6.08e ⁻⁸	0.00257	ENSSSAG000000084335	Intron 1
	LND.DF	-	-	-	-	-	-	-
HT	Time	10	473868	-7.176399	8.80e ⁻⁸	0.00373	ENSSSAG00000105432	Intergenic
	DF	10	536348	2.5754604	4.66 e ⁻⁸	0.00099	ENSSSAG00000105432	Intergenic
	LND	-	-	-	-	-	-	-
	LL.DF	2	39179344	-5.984758	4.03 e ⁻⁷	0.00709	ENSSSAG000000073815	Other intron
	LND.DF	-	-	-	-	-	-	-

Table 3-5 Number of putative differently methylated cytosines (DMC) (omitted FDR threshold) and genes overlapped by DMC (DMC_{genes}) for each treatment groups, characterised as hypo- or hyper-methylated compared to control conditions (LL.CF). In this LT is low temperature, HT is high temperature, DF is feeding in the perceived day, CF is continuous feeding, LND is light dark photoperiod, and LL is continuous light.

		Hyper		Hypo		Total	
		DMC	DMC _{genes}	DMC	Genes	DMC	DMC _{genes}
LT	Time	51	51	82	78	133	128
	LND	87	86	57	57	144	142
	DF	63	63	67	64	130	126
	LL.DF	77	76	62	61	139	134
	LND.DF	99	99	75	74	174	172
HT	Time	83	77	39	38	122	113
	LND	75	70	55	53	130	121
	DF	92	92	66	65	158	156
	LL.DF	49	48	82	82	131	128
	LND.DF	44	43	46	46	90	89

3.4.4.1.DMC characterisation

Putative DMC_{800dd} showed some variability in genomic annotation between treatments (Figure 3-5), but this was not significantly different when compared to each other or all CpG sites (Chi-square test goodness of fit: $p > 0.05$) (Appendix B.4). Similarly, there was no apparent difference in the portion of genomic features when compared between hypo- and hyper-methylated putative DMC_{800dd} within each treatment group (Chi-square test for association, $p > 0.05$) (Appendix B.5). The number of DMC_{genes} were similar to the number of DMC themselves (Table 3-5), as the majority of DMC_{genes} contained only a single DMC (Appendix B.12). While DMC_{genes} were, in general, unique to treatment groups, those exposed to day feeding, shared a greater proportion of DMC_{genes} (Figure 3-5). In line with this, the LND

and LL.DF group showed the fewest shared set of DMC_{genes}. Notably, a single DMC_{gene}, in both the high and low temperature group, was shared across all treatments. For high temperature this was *ENSSSAG00000057174*, and low temperature *ENSSSAG00000105432*. However, no indication of functional enrichment was found in any gene set.

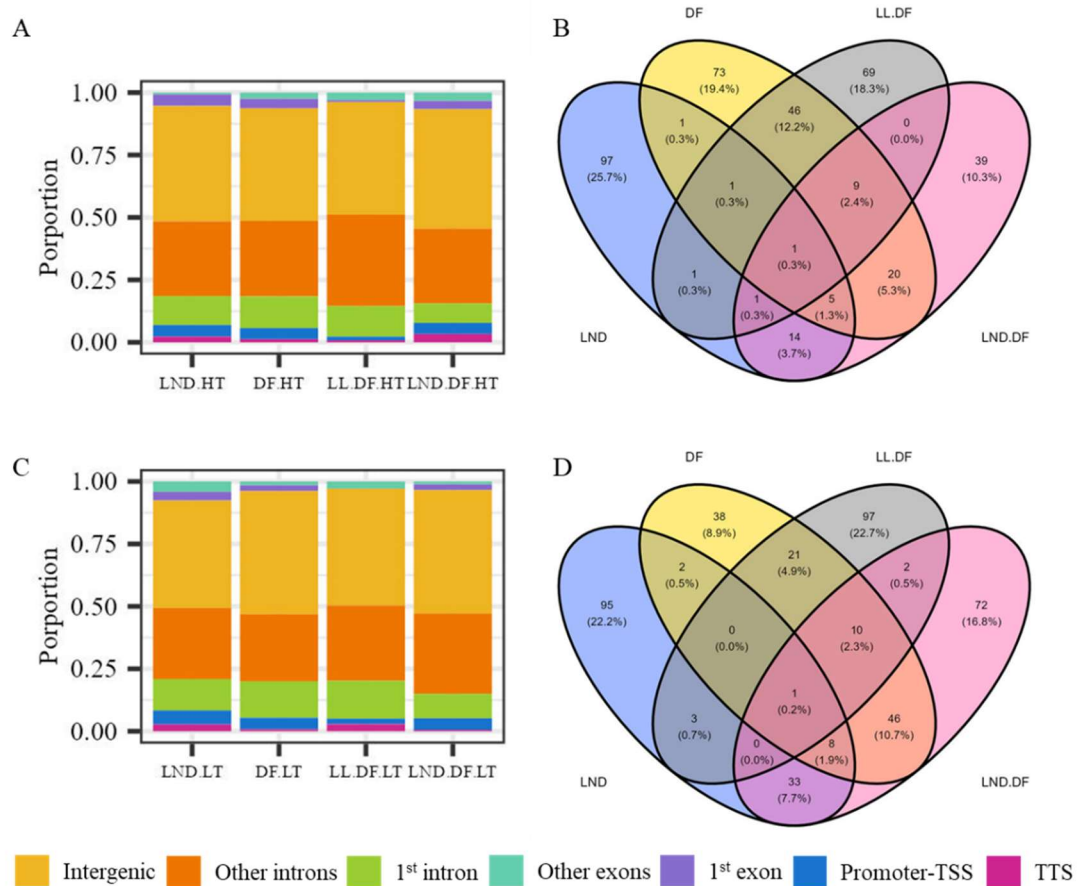


Figure 3-5 Genomic annotation of DMC₈₀₀ per treatment for low (A) and high (C) temperature groups, as well as Venn diagram of DMC_{genes} for low (B) and high (D) temperature groups. In this, DF is feeding in the perceived day, CF is continuous feeding, LND is light dark photoperiod, and LL is continuous light.

3.4.5. Transcriptomics

3.4.5.1. RNA-seq libraries and background gene set

On average 54,412,873.35 (s.e 1,279,681.80) reads were obtained across samples before filtering. After filtering, approximately 89% of reads were retained, with an average duplication rate of 29.15%. Sequencing information of all samples can be seen in Appendix B. 6. A total of 69,389 genes were available for analysis.

3.4.5.2. Photoperiod and feeding regime induced expression changes

Dimension reduction revealed strong clustering by treatment (Figure 3-4). At high temperature, groups split broadly into two, in line with photoperiod and feeding regime. Specifically, samples under LL.CF fell low on dimension 2 and high on dimension 1, with the opposite true of those under LND.DF. Fish exposed to LL.DF were split between the formed clusters. In contrast, in the low temperature group, there was formation of three clear clusters. While this was largely in line with photoperiod treatment, as with the high temperature, a third group fell between these “LL” and “LND” clusters and was a mixture of all three treatments.

A substantially greater number of differently expressed genes through time (DEG_{time}) were identified for the low compared to high temperature group (Table 3-6, Appendices B. 17-B. 18). Overall gene expression changes between treatment conditions at 800dd were greater at high compared to low temperature (Table 3-6, Appendices B. 17-B. 18). A greater number of DEG_{800dd} were found in response to photoperiod compared to feeding regime manipulation. At high temperature, the combination of cyclic photoperiod and feeding regime showed the greatest difference in gene expression (Table 3-6).

Table 3-6 Number of DEG for each treatment groups, characterised as up- or down-regulated compared to control conditions (LL.CF). In this LT is low temperature, HT is high temperature, DF is feeding in the perceived day, CF is continuous feeding, LND is light dark photoperiod, and LL is continuous light.

		Up-regulated	Down-regulated	Total
LT	<i>Time</i>	483	1501	1,984
	<i>LND</i>	4	2	6
	<i>DF</i>	0	0	0
	<i>LL.DF</i>	0	0	0
	<i>LND.DF</i>	1	3	4
HT	<i>Time</i>	1	3	4
	<i>LND</i>	216	823	1,039
	<i>DF</i>	4	0	4
	<i>LL.DF</i>	3	0	3
	<i>LND.DF</i>	1,668	669	2,367

3.4.5.3. DEG characterisation

GO terms associated with high temperature DEG_{time} can be seen in Appendix B. 7. Though no enrichment was found for up-regulated DEG_{time} in the low temperature group, several

pathways were identified for the down-regulated genes (Appendix B. 16). These related to carbon and amino acid metabolism, signal transduction in environmental information processing, transportation and catabolism within cellular processes, gonadotropin-releasing (GnRH) signalling pathway and salmonella infection pathway.

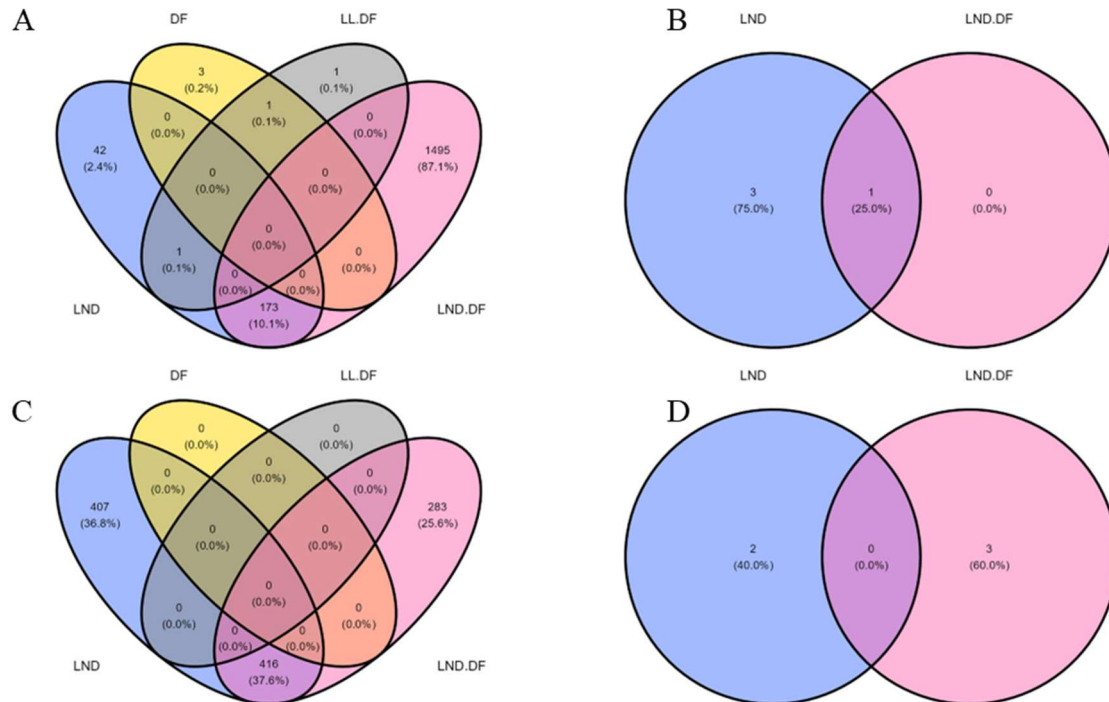


Figure 3-6 Venn diagram for up- (A and B) and down- (C and D) regulated DEGs for high (A and B) and low (C and D) temperature groups. In this, DF is feeding in the perceived day, CF is continuous feeding, LND is light dark photoperiod, and LL is continuous light.

Overall, there were no DEGs universally affected by environmental manipulation (Figure 3-6). At low temperature group, a single gene (*LYSC2*) was shared between LND and LND.DF, where it was found to be up-regulated in both. Detailed information on the other DEGs in these groups can be seen in Appendix B. 7. At high temperature, only a single gene (*ENSSSAG00000093579*) was up-regulated and shared between DF and LL.DF. A further gene (*ENSSSAG00000114681*) was shared between LND and LL.DF, again up-regulated in both (Appendix B. 7). While a greater number of DEGs were identified for LND and LND.DF treatment groups, they also shared a portion of these.

Although no enrichment was identified in down-regulated DEGs at high temperature, several pathways were identified in up-regulated genes (Figure 3-7). Specifically, DEGs in response of LND only showed enrichment for adrenergic signalling in cardiomyocytes, a part of the circulatory system. It is important to note, only a very small proportion of genes in this

pathway were affected. DEG_{800dd} in response to combined effect of LND and DF were related to transcription, translation as well as folding, sorting and degradation of genetic information. Lastly, those up-regulated in response to LND alone or in combination with DF were in pathways related to translation as well as folding, sorting and degradation.

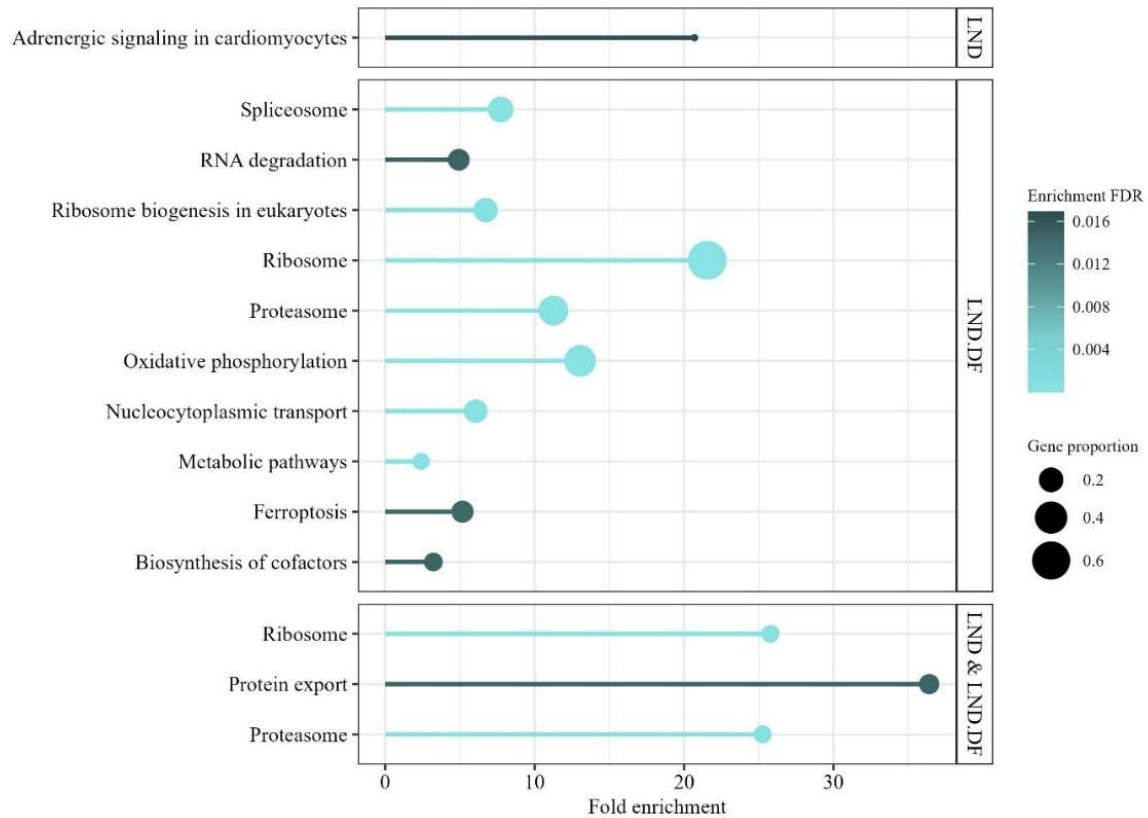


Figure 3-7 Visualisation of KEGG enrichment for DEG_{800dd} up-regulated in treatment groups relative to baseline conditions. Coloured by enrichment false discovery rate (FDR) and the size of dots represent the proportion of genes in a pathway that were differentially expressed. In this, DF is feeding in the perceived day, CF is continuous feeding, LND is light dark photoperiod, and LL is continuous light.

3.4.6. Integration between differential methylation and expression

Of filtered CpGs, correlations calculated across all samples between methylation proportion and normalized gene expression of overlapping genes could not be calculated in 12,062 sites, or 28.48 % (Figure 3-8), due to non-variable gene expression. Of those where a correlation was calculable, the vast majority (94.58%) showed a non-significant association at 5% significance. For baseline and experimental groups, the proportion of significant correlations was largely similar, with the relative proportion of non-calculable and non-significant correlations fluctuating (Appendices B.19-B.20). Within this, there was a roughly equal split between positive and negative correlations. Across all samples, the genomic annotation of CpG sites with significant and non-significant correlation was largely similar (Figure 3-8). The exception

was positive significant correlations, where there was a slight excess of CpG sites annotated to promoters-TSS. The distribution between genomic features in other groups is largely the same as observed across all samples (Appendix B.21).

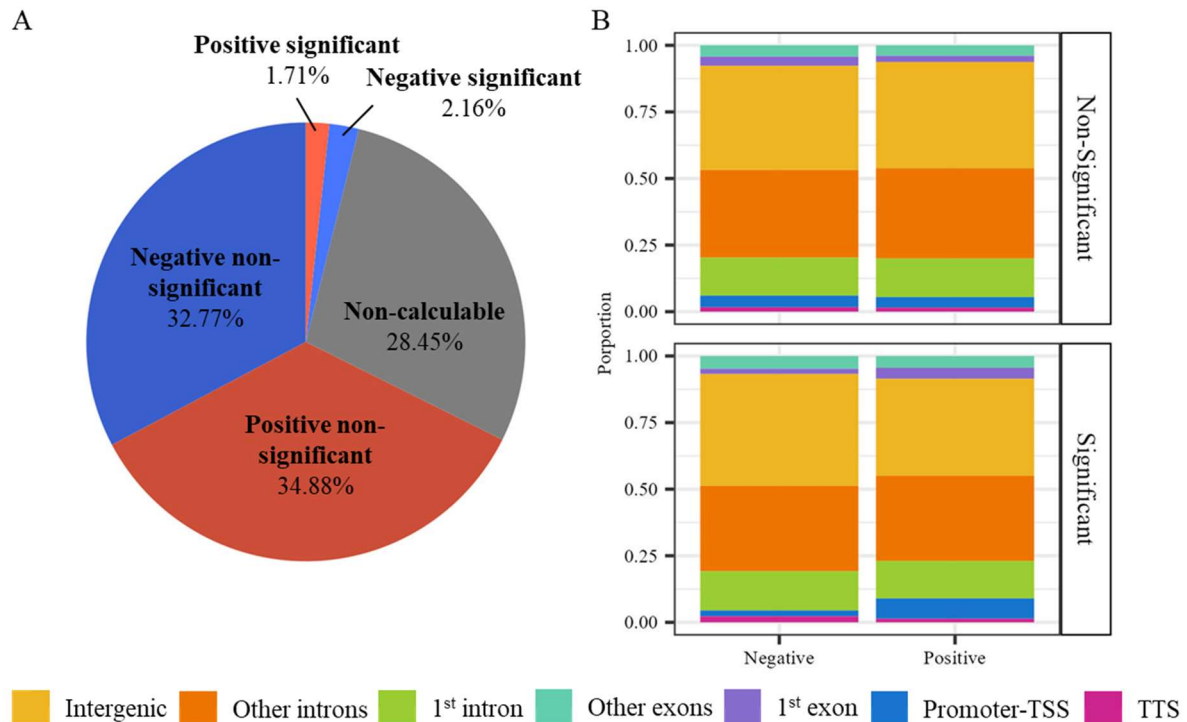


Figure 3-8 A) Pie chart demonstrating the percentage of filtered CpG sites where the proportionate methylation was in significant, non-significant or non-calculable correlation with overlapping genes normalized (FPKM) gene expression across all samples (n=37). Correlations are split based on the direction of change, where red is positive and blue negative. Pearsons correlation at 5% significance. B) Genomic annotation of CpG-gene combinations in significant and non-significant correlations, split by direction of change.

Based on average CpG site methylation proportion and normalized gene expression of overlapping genes, calculated across all samples as well as individual groups, correlations were weak ($-0.035 < r < 0.114$), though some were significant (Figure 3-9). Specifically, there was little indication of meaningful correlation for CpG sites located in intergenic regions ($r = 0.004-0.009$), the 1st intron ($r = -0.015-0.009$), other-exons ($r = 0.025-0.033$), or TTS ($r = 0.024-0.056$). However, weak but significant positive correlations were seen between gene expression and methylation in the 1st exon ($r = 0.030-0.100$) or promoter-TSS ($r = 0.076-0.1144$). In contrast, weak but significant negative correlation was seen in other-introns ($r = -0.035-(-0.018)$) (Figure 3-9). Of note, while correlations in groups at 800dd largely aligned with that seen across all samples, the baseline appeared as an outlier, often showing weaker correlations. Scant overlap between DMC and DEG were observed.

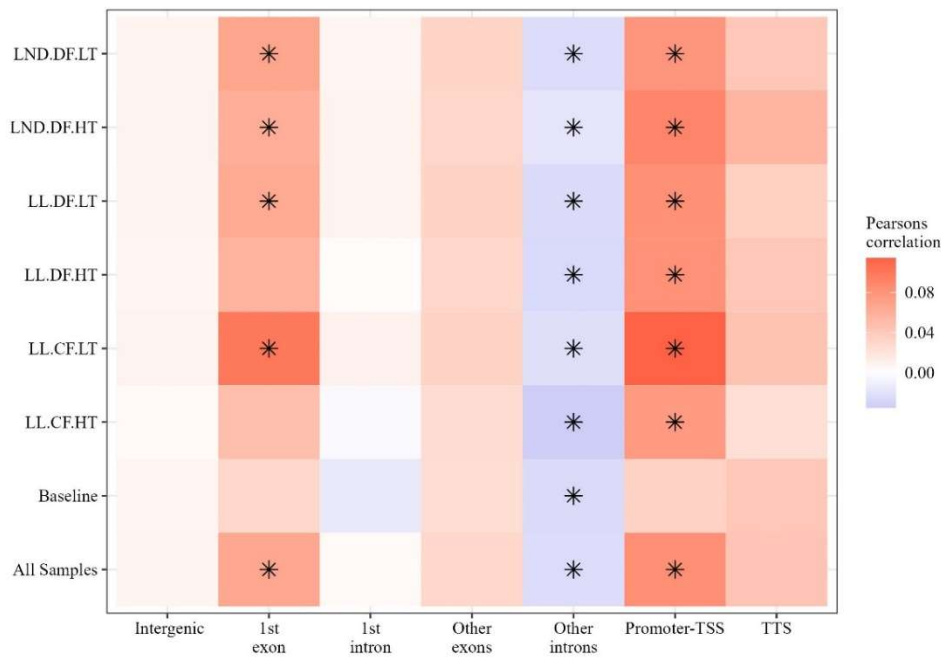


Figure 3-9 Heatmap displaying of Pearson's correlation between average methylation proportion of filtered CpG sites with overlapping average normalised (FPKM) gene expression levels, split by genomic feature. This was performed across all samples, as well as the baseline and each treatment groups separately. * indicates significance at $p < 0.05$. In this, LT is low temperature, HT is high temperature, DF is feeding in the perceived day, CF is continuous feeding, LND is light dark photoperiod, and LL is continuous light.

3.5.Discussion

3.5.1.Characterisation of CpG methylation

In this study, while both the minority of sequenced cytosines were located at CpG sites and the minority of cytosines were methylated, the majority of methylated sites were in a CpG context, falling in line with current understanding (Eirin-Lopez and Putnam, 2019). Despite this, across the genome, a pattern of bi-modality was seen in CpG methylation (Figure 3-3). This agrees with previous studies which have linked CpG methylation to the density of CpG sites. Specifically, while methylation is predominantly found in a CpG context, genomic regions which have a high density of CpG sites are frequently found to be unmethylated. Such regions include CpG islands (CpGi) which are protected from methylation via various mechanisms (Long *et al.*, 2016; Ambrosi, Manzo and Baubec, 2017; Clark and Rager, 2020).

While across genomic features similar bi-modal distributions were found, differences can be observed (Figure 3-3). Similarly, between CpG sites with fixed methylation or unmethylation, the proportions of genomic features differed (Figure 3-2). Firstly, as reported previously (Anastasiadi, Esteve-Codina and Piferrer, 2018; Uren Webster *et al.*, 2018; Mukiibi *et al.*, 2022),

CpGs located in the promoter-TTS showed a greater peak at 0 than 1 for proportionate methylation (Figure 3-3). Given that methylation at gene promoters is generally accepted to be transcriptionally repressive (Jjingo *et al.*, 2012), this pattern reflects conserved divergence of promoters based on their CpG density (Elango and Yi, 2008). Specifically, two groups have evolved, (1) broadly expressed, CpG dense, and lowly methylated housekeeping genes, and (2) CpG depleted, highly methylated, tissue-specific genes (Elango and Yi, 2008). As the majority of genes were overlapped by a single CpG (Appendix B. 12), it was not possible to investigate if the bi-modality CpG site methylation was related to their density. However, CpG sites annotated to promoters-TSS formed a greater proportion of unmethylated CpGs (Figure 3-3). This aligns with previous studies (Uren Webster *et al.*, 2018; Mukiibi *et al.*, 2022) and the general observation of a low methylation state of TSS (Jjingo *et al.*, 2012) as well as the fact that most promoters (~60%) are overlapped by methylation resistant CpGi (Kulis *et al.*, 2013; Ambrosi, Manzo and Baubec, 2017).

By contrast, CpG sites annotated to intergenic or gene body regions (other-exons, other-introns, and 1st introns), as well as to TTS showed, a higher level of methylation (Figure 3-3). In addition, intergenic CpGs as well as those assigned to other-introns constituted a smaller portion of sites found to be always-unmethylated (Figure 3-2). Methylation of the gene body and intergenic regions has readily been observed (Anastasiadi, Esteve-Codina and Piferrer, 2018; Mukiibi *et al.*, 2022), falling in line with their generally low level of CpG density (Brenet *et al.*, 2011). Functionally this has been related to the suppression of transposable elements, alternative TSS, and non-coding RNAs, which is important for genomic integrity and stability (Kulis *et al.*, 2013). While others have also recorded higher levels of methylation at TTS (Jjingo *et al.*, 2012; Anastasiadi, Esteve-Codina and Piferrer, 2018; Mukiibi *et al.*, 2022), exploration into why this is observed is scant. It has, however, been suggested that induced hypomethylation at TTS generates aberrant transcriptional termination (Shirai *et al.*, 2022).

Distinct from other gene body CpGs, those located in the 1st exon showed a tendency to be lowly methylated (Figure 3-2 and Figure 3-3) (Brenet *et al.*, 2011). Previously, methylation at the 1st exon/promoter boundary has been found to have a strong inverse relationship with gene transcription by Brenet *et al.* (2011). The authors also found that even lowly expressed genes had unmethylated 1st exons, taking this to suggest that the process of transcription

initiation itself protects these CpGs from becoming methylated. However, it could be that low 1st exon methylation is due to their physical proximity to TSS-promoters. Given that 90% of 1st exon CpGs fall within 113 to 2,463 bp downstream of TSS-promoter (Appendix B. 13), it is possible that CpGs (or CpG dense regions) that are regularly found at promoters and maintain low methylation, extend downstream into the 1st exon (Hartono, Korf and Chédin, 2015). If so, this may in part explain the absence of highly methylated 1st exons.

3.5.2. Impact of environmental manipulation of DNAm

Overall, at a genomic scale, environmental manipulation had a limited impact on methylation, but it can still be seen that fish held at high temperature demonstrated a lower proportion of CpG sites with variable methylation. Instead, there is a greater amount of cytosines with fixed methylation or unmethylation (Figure 3-2). An inverse relationship between vertebrate body temperature and DNAm has previously been identified on an evolutionary time scale due to the increased rate of deamination of methylated cytosines (Varriale and Bernardi, 2006; Varriale, 2014; Metzger and Schulte, 2017). Studies have also identified decreases in global methylation due to acute increases in environmental temperature in fishes (Burgerhout *et al.*, 2017; Metzger and Schulte, 2017; Beemelmanns, Ribas, *et al.*, 2021).

While this effect is expected to be more pronounced during embryonic and early life development (Anastasiadi, Díaz and Piferrer, 2017; Kho *et al.*, 2024), exposure to temperature manipulation during later life has also resulted in decreased DNAm (Beemelmanns, Ribas, *et al.*, 2021). Equally, while these studies have focused on temperature changes of greater magnitude, even small increases (~2°C) have been found to alter global methylation if applied in particularly sensitive developmental stages (Anastasiadi, Díaz and Piferrer, 2017; Kho *et al.*, 2024). These results may help explain the greater proportion of always-unmethylated cytosines that were observed at high temperature. However, these samples were also seen as having more always-methylated cytosines. It is therefore worth mentioning that contrasting results have been found. For example, Metzger and Schulte (2017) reported that any change in environmental temperature resulted in increased genomic methylation due to a greater portion of highly methylated cytosines, and Beemelmanns, Zanuzzo, *et al.* (2021) reported that increased temperature led to both hyper- and hypo-methylation.

Differences in global methylation were also seen between treatments within temperatures. While this was only slight at low temperature (slight excess of fixed methylation in LL.DF), it was more apparent at high temperature. Specifically, those under LL.CF demonstrated a lower proportion of CpG sites with variable methylation (Figure 3-2). Information on photoperiodic or feeding regime manipulation on DNA methylation is limited. Yet, Kho *et al.* (2024) similarly found that increased photoperiodicity was associated with a non-significant downward trend in global levels of methylation in Atlantic herring, *Clupea harengus*. Indications of hypo-methylation in line with increased or continuous photoperiod have also been seen in comparison between either wild caught or semi-naturally reared salmonids to their wild caught counterparts. In such comparisons, the former would be akin to the LL.CF treatments, and later LND.DF. Considering the direction of change, increased portion of always-unmethylated CpG sites of LL.CF.HT is consistent with the methylation patterns reported for hatchery rearing by some (Gavery *et al.*, 2019; Wellband *et al.*, 2021) but contrasts with others (Le Luyer *et al.*, 2017).

Though there were some treatment related differences at the level of global methylation (Figure 3-2 and Figure 3-3), at the finer-scale very few DMCs were identified (Table 3-4), and while relaxing the FDR threshold did reveal putative DMCs (Table 3-5), samples show no indication of clustering based on proportion of methylation (Figure 3-4). In agreement Kho *et al.* (2024) did not observe clustering of Atlantic herring, *C. harengus*, larvae by photoperiod treatments. However, these results contrast strongly with previous research on the impact of hatchery rearing, where fish were grouped by rearing environment and not by genetic origin (Le Luyer *et al.*, 2017; Gavery *et al.*, 2018). Such studies were also successful in identifying DMCs between treatment groups, often highlighting shared sets of differently methylated regions (Gavery *et al.*, 2018) or genes (Wellband *et al.*, 2021).

This lack of DMCs may be a result of the time in which environmental manipulation was applied. While other studies have identified clear changes in response to rearing environment (Blouin *et al.*, 2010; Gavery *et al.*, 2019; Rodriguez Barreto *et al.*, 2019; Wellband *et al.*, 2021), as well as specific environmental variables (Craig and Moon, 2013; Burgerhout *et al.*, 2017; Moghadam *et al.*, 2017; Veron *et al.*, 2018; Robinson *et al.*, 2019; Kelly *et al.*, 2020), these have often been applied during or over a period encompassing early life development, that is

embryonic and larval stages up to first feeding (Anastasiadi, Díaz and Piferrer, 2017). Importantly, during this time, genomic patterns are thought to be more sensitive or susceptible as the methylome is still being established (Granada *et al.*, 2018; Clark and Rager, 2020). It could be that because environmental manipulation applied here was outside these developmental periods, the changes caused are instead more transient, not being maintained through cellular divisions and instead passively eroded and replaced. To counter this, however, incorporation of environmental stimuli into the methylome has been seen at later life stages (e.g. Rodriguez Barreto *et al.*, 2019), and was observed to be retained through time (Leiva *et al.*, 2020).

In addition, the tissue of choice may have contributed to the lack of DMC identified. In line with DNAm's role in cellular differentiation, contrasting methylation patterns have been seen across cell types (Lee, Hore and Reik, 2014; Yang *et al.*, 2016). Use of homogenous tissues is therefore often recommended for DNAm analysis (Gavery *et al.*, 2018; Wellband *et al.*, 2021). Consequently, despite brain tissue being chosen due to its role in detection, integration, and relaying of environmental information during freshwater development (Migaud *et al.*, 2007; Falcón *et al.*, 2010; Migaud, Davie and Taylor, 2010), as well as being identified as having a high number of DMCs (Baerwald *et al.*, 2016), differential methylation signals between its heterogeneous cell types and brain regions may have masked detection of DMC.

Alternatively, the characteristics of the environmental manipulation applied may not have been sufficient to illicit meaningful changes between treatment groups. For example, while the duration of light exposure was manipulated, light intensity, also a critical factor in eliciting a physiological response (Migaud *et al.*, 2007; Vera *et al.*, 2010), was not. Equally, while the environmental parameters manipulated here did not detect large scale differences in DNAm, if the holistic RAS environment was studied instead (like Le Luyer *et al.* (2017)) the combinatory effect of many variables may have done so. It may be worth taking such an approach in the future studies.

It is worth noting that across all filtered CpG sites, and consequently in all groups of putative DMCs, there was no indication of significant enrichment in a particular pathway. This finding may relate to the relatively low number of genes that the background set of CpG sites overlapped (8,322), though enrichment analysis is sensitive to several factors which could also

have had an effect (Geistlinger *et al.*, 2021). In addition, while whole genome bi-sulphite sequencing is considered the 'gold-standard' approach for DNAm studies, RRBS targets only a fraction of the genome, specifically enriching for CpG dense regions. As a consequence, it may have missed critical information (Kurdyukov and Bullock, 2016). Furthermore, investigation into methylation patterns in non-model species, as well as fish in particular, is limited compared to mammals. Likewise, many available analytical approaches have been developed for mammalian models and because of notable differences between mammalian and non-mammalian vertebrate methylomes, relying on such resources may have affected the results (Long *et al.*, 2013; Wellband *et al.*, 2021).

3.5.3. Transcriptomic response to environmental manipulation

In contrast to the results of DNAm, clear treatment effects were seen in gene expression. While samples broadly clustered by experimental treatment, this was principally in line with photoperiod (Figure 3-4). These differences in global expression were underpinned by a number of differently expressed genes (DEG), mainly in response to cyclic photoperiod rather than cyclic feeding (Table 3-6), with enrichment analysis indicating changes in metabolic activity relative to LL.CF (Figure 3-7). Considering this energy metabolism in teleost fish brains principally uses exogenous sources of glucose, with the concentration in the brain correlating with that of circulating/plasma levels (Soengas *et al.*, 1998; Soengas and Aldegunde, 2002). In line with this, the use of continuous light and feed provision typically employed in aquaculture has been previously linked to increases in carbohydrate utilisation and enhanced growth rates observed under such treatments (Churova *et al.*, 2020; Kuznetsova, Rodin, Shulgina, Krupnova, Kuritsyn, *et al.*, 2023; Kuznetsova, Rodin, Shulgina, Krupnova, Kuritsyn, *et al.*, 2023). However, in periods of feed deprivation or when glucose and glycogen stores are depleted, there are indications that alternative energy sources, such as fatty acids, are utilized (Soengas and Aldegunde, 2002). Considering this, results indicating a change in metabolic activity (Figure 3-7) may reflect night-time fasting in the LND treatment groups relative to fish held under LL photoperiod. In support, Soengas *et al.* (1998) demonstrated that administration of melatonin drove decreased and increased glucose and fatty acid derivatives concentration, respectively, in a dose-dependent manner.

Additionally, results indicated increased genetic processing, protein synthesis and cellular turnover in response to LND in isolation or combination of day feeding (Figure 3-7). Possible functions of this within the brain are unclear, with little information available in teleosts. However, given that neuronal plasticity can be shaped by environmental factors during development (Ebbesson and Braithwaite, 2012), it may be that photoperiod manipulation triggered differences in neural structure. For example, comparison between wild caught and hatchery reared salmonids have demonstrated poorer cognitive development and performance in the latter, relating this to the relative stability and low enrichment of captive environments (Kihlslinger and Nevitt, 2006; Ebbesson and Braithwaite, 2012; Mes *et al.*, 2018). Furthermore, it was found that under continuous light, relative to simulated natural photoperiods, neural innervation of the pre-optic area, certain neurotransmitter activity, and neurogenesis were inhibited during parr to smolt development (Ebbesson *et al.*, 2007, 2011).

Much of the neural plasticity and development of anadromous salmonids during freshwater development can, however, become confounded by smoltification (Ebbesson *et al.*, 2007; Ebbesson and Braithwaite, 2012). Given the evidence of transcription changes in response to smoltification in other tissues (Seear *et al.*, 2010; Robertson and McCormick, 2012), it is unfortunate that we were unable to assess this fully under the current study. Yet, while some smolt characteristics can develop in the absence of a zeitgeber, the lack of key photoperiod cues suggests that smoltification is less likely to have occurred (Strand, Hazlerigg and Jørgensen, 2018). Future studies should directly consider smoltification state in studying epigenetic changes during freshwater development, especially in instances of photoperiod and temperature manipulation.

It is worth noting that treatment effects were clearer at high compared to low temperature treatments. In agreement, previous studies have highlighted how lower temperatures can dampen the photoperiodic effects on transcription. For example, Prokkola *et al.* (2018) found that Arctic charr, *Salvelinus alpinus*, reared at a lower temperature exhibited reduced magnitude of circadian rhythms in the hepatic transcriptome. Similarly, Nisembaum *et al.* (2020) found that daily oscillations of melatonin were smaller when Atlantic salmon was reared at a lower temperature, though this was also modulated by the prevailing photoperiod.

3.5.3.1. Differential transcription through time

In the results presented here, two contrasting patterns emerged. Firstly, at low temperature differences through time were observed, though none were really seen between treatment groups (Table 3-6). Enrichment analysis for these low temperature DEG indicated decreased expression of genes related to energy (carbon and amino acid) metabolism, environmental signalling pathways, autophagy, lysosomes, phagosomes, and reproductive endocrinology (Appendix B. 16). In contrast, very few DEG through time were observed for high temperature, though differences between treatments were apparent (Table 3-6). The cause of this is still unclear. However, it is important to highlight that due to the failure of degree days to adequately account for differences in growth between temperature regimes (Appendix B. 3), there was no 'true' baseline for the high temperature group. Specifically, at high temperature, these DEG were identified through a comparison between the baseline, which was held at low temperature and exposed to continuous light and feed, to the same photoperiod and feeding regime at 800dd, but at high temperature. Therefore, DEGs identified between high temperature treatments may, in part, have been driven by the heightened temperature experienced, especially if any interactive effect with photoperiod was present. Furthermore, in terms of actual days, after 800dd there was a three-week difference between the low and high temperature groups. This temporal deficit and earlier sampling of high temperature groups may have further influenced the low number of DEG through time. Further investigation into the effects of temperature treatment on gene expression and DNAm in salmonids may help elucidate whether this result is a biological one or artifact of experimental design, analytical approach, and their limitations.

3.5.4. Correlations between CpG methylation and gene expression

Across all CpG site, a significant correlation between methylation proportion and the expression of overlapping genes were observed only in a minority of cases, both overall and when split by treatment group (Figure 3-8). High levels of non-significant correlations have previously been found by Mukiibi *et al.* (2022) and Leiva *et al.* (2021) and was attributed to the bulk nature of RRBS and RNA-seq analysis, especially in the context of diverse cell types within tissues. Equally, the regulatory role of DNAm has been suggested as limited to the extremes, that is when CpGs are fully methylated or unmethylated, whereas genes of

intermediate methylation are governed by other mechanisms (Brenet *et al.*, 2011; Lou *et al.*, 2014). Therefore, finding few significant correlations may be related to the proportion of CpG sites recorded as having variable methylation between samples (Figure 3-2).

Of the significant correlations, an approximately equal proportion of positive and negative relationships were seen, with similar genomic annotation seen across all CpGs (Figure 3-8). However, when average methylation and expression levels were considered instead, these overarching correlations differed between genomic features (Figure 3-9). Specifically, a weak but significant positive correlation was recorded for CpGs falling in the promoter-TSS. This contrasts with the canonical model of promoter methylation repressing gene expression, as well as previous studies which have identified weak but significant negative correlations between the two (Anastasiadi, Esteve-Codina and Piferrer, 2018; Uren Webster *et al.*, 2018; Mukiibi *et al.*, 2022). Under this model, DNase of promoter CpGs regulates expression by mediating the action of transcription factors (TF) (Zhu, Wang and Qian, 2016). This can occur (1) directly by methylation inhibiting TF binding, or (2) indirectly by recruiting TF with methyl-binding-domain (MBD) that secondarily alter chromatin state. Other mechanisms for the expressive impacts of promoter methylation have also been suggested, such as steric interference (Siegfried and Simon, 2010).

However, with increasing research integrating expression and methylation data, this picture has become more complex and context dependent (Ambrosi, Manzo and Baubec, 2017; de Mendoza *et al.*, 2022). For example, it has been suggested that this inhibitory effect of promoter methylation on gene expression is restricted to those rich in CpGs or containing CpGi (Weber *et al.*, 2007). As described above, these are typically housekeeping genes which are typically both hypomethylated and expressed, as well as often being conserved across taxa and/or tissues. In contrast, tissue-specific promoters have low CpG density and tend towards hypermethylation. Importantly, tissue specific differentially methylated regions (tDMR) have repeatedly been positively correlated with gene expression (Davies *et al.*, 2012; Wan *et al.*, 2015; de Mendoza *et al.*, 2022) and identified to be enriched in promoters (Wan *et al.*, 2015; Anastasiadi, Esteve-Codina and Piferrer, 2018). In line with this, de Mendoza *et al.* (2022) recently found that while induced methylation drove decreased gene expression in a large portion of human genome, many promoters escaped this pattern. This observation was

attributed to the action of specific TF and their binding affinities. Specifically, genes whose expression increases under promoter methylation do so due to the repelling of transcriptional repressors containing methyl-sensitive binding sites. Furthermore, promoters that are more likely to be repressed by methylation contain an over-representation of TF binding motifs, high CpG density or a wider unmethylated region. While these findings support the presence of positive correlations between promoter methylation and gene expression, it would be of interest to see if their characteristics (e.g. CpG density or tissue specificity) also align with these previous studies.

Considering gene body methylation, correlations between average methylation and expression of introns and exons showed variable results with both positive and negative correlations identified (Figure 3-8, Figure 3-9). Previous studies have considered the impact of gene body methylation on gene expression collectively and similarly reported both positive and negative correlations (Rountree and Selker, 1997; Lorincz *et al.*, 2004; Ball *et al.*, 2009; Lou *et al.*, 2014; Uren Webster *et al.*, 2018). While the exact molecular mechanism underpinning these significant correlations is less well understood, explanations have been put forward. For example, methylation in gene bodies has been positively correlated with expression due to repression of spurious transcription, as well as being purely a by-product of *de novo* methylation during transcription itself (Ball *et al.*, 2009; Jjingo *et al.*, 2012). By contrast, negative correlations have also been proposed due to inhibition of transcription elongation, or recruitment of transcriptional repressors (Rountree and Selker, 1997; Lorincz *et al.*, 2004; Lou *et al.*, 2014). Some of this variability is in part due to the CpG density of methylated regions. Specifically, low density regions are constitutively methylated, whereas higher density regions have more variable methylation, with the function of CpG methylation varying with genomic location (Brenet *et al.*, 2011).

Correlations were also specific to gene body features, in agreement with previous studies (e.g. Brenet *et al.*, (2011)). However, the direction of change in gene expression associated with methylation of these regions differed from what has been previously reported. For example, previous research identified strong inverse correlations with gene expression with the 1st exon (Ball *et al.*, 2009; Brenet *et al.*, 2011; Anastasiadi, Esteve-Codina and Piferrer, 2018; Mukiibi *et al.*, 2022) or 1st intron (Anastasiadi, Esteve-Codina and Piferrer, 2018; Mukiibi *et al.*, 2022). In

contrast, the results presented here showed non-significant correlations for CpGs in other-exons and 1st introns, weak but positive for the 1st exon, and weak but negative correlations for other-introns (Figure 3-9). Considering this, previous studies have found a portion of CpGs annotated to the 1st exon in positive methylation-gene expression correlations, implicating this in tissue specific expression patterns or found to overlap tDMR (Davies *et al.*, 2012; Wan *et al.*, 2015). This may, in part, help explain the positive correlation observed for the 1st exon. In contrast, the negative correlation between other-introns methylation and expression could relate to the presence of distal regulatory regions (Blattler *et al.*, 2014; Angeloni and Bogdanovic, 2019). It is of note that variability in correlative relationship between DNAm and gene expression reported previously, as well as in this study, may be influenced by the tissues type, analytical approach chosen, and sequencing technologies applied (Brenet *et al.*, 2011; Lou *et al.*, 2014).

Despite these general correlations, given the weak indications of differential methylation, there was scant overlap between DMC and DEG. This suggests any influence of environmental manipulation on DNAm had limited functional impact on gene expression. Conversely, while environmental manipulation appears to have driven changes in gene expression other molecular mechanisms may have underpinned this. In support, Harvey *et al.* (2024) found transcriptional changes associated with photoperiod manipulation were not accompanied by changes in DNAm, but rather showed links to specific histone marks or modifications (Iversen *et al.*, 2020, 2021).

3.6.Conclusion

Here, characteristics of the brain methylome were investigated, as well as any changes because of environmental manipulation. While differences were seen in methylation status between genomic features (which were largely consistent between treatments), across all CpG sites samples did not cluster by treatment and only a few differently methylated cytosines (DMC) were identified. In contrast, gene expression data showed clear clustering of samples by photoperiod and feeding regime treatment. These differences were underpinned by differentially expressed genes (DEG), with changes more apparent when treatments were applied at high temperature. Weak but significant overarching correlations were identified in some genomic features. However, little overlap between DMC and DEG indicate that

methylation changes resulting from environmental manipulation was not strongly associated with altered gene transcription.

Chapter 4-Estimating Genotype-environment interactions, Three-Months Post-Saltwater Transfer, in Atlantic Salmon (*Salmo salar*) Reared in Alternative Freshwater Systems

4.1. Abstract

While net-pens in freshwater (FW) lochs have previously been used up to smoltification, there is interest in using recirculating aquaculture systems (RAS) for Atlantic Salmon, *Salmo salar*, aquaculture. Indications that RAS impacts post-saltwater (SW) transfer performance has implications for its use. Gaining deeper understanding as to how RAS affects fish throughout the production cycle is necessary. Working within a commercial setting, a population of young salmon were co-reared for approximately nine-months, before separation for about eight weeks between a RAS or loch system. After this, both were transferred to a shared SW cage site. Phenotypic measurements were taken at the end of FW-rearing and at three-months post-SW transfer. Fish were genotyped on a ~65K SNP array. Results indicate while RAS-reared smolts were smaller at the end of FW-rearing, they demonstrated enhanced growth rate post-SW transfer. Additionally, in both FW and SW, RAS-reared smolts had lower trait variance, with indications of a sex difference in growth in the loch population. Genetic parameter estimates revealed heritability in the loch population increased from FW to SW, while the opposite was observed for RAS-reared fish. While GxE in SW phenotypic traits were weak, moderate-to-strong estimates were found for growth rates. Furthermore, genetic correlations of growth traits between FW and SW were generally high, at the exception of whole-body weight in the loch population. Results suggests that FW-rearing environment impacts SW growth performance as well as genomic regulation of these traits. Consideration of this in management and selection practices may improve production efficiency.

4.2.Introduction

Traditionally, commercial production of Atlantic salmon (*Salmo salar*, L. 1758) within the UK has involved rearing fish in hatcheries before transfer to freshwater (FW) net-pens in lochs for smoltification (Ellis *et al.*, 2016; Clarke and Bostock, 2017). This is a key stage of development where fish acquire morphological, physiological, and behavioural adaptations that facilitate their anadromous life cycle (Stefansson *et al.*, 2008; McCormick, 2012). After this stage, post-smolts are typically transferred to saltwater (SW) cages for a grow-out period until they reach harvest size at ~3-5kg (Ellis *et al.*, 2016). However, within the industry, there is an increasing trend to shift away from the use of FW lochs to land-based systems, such as recirculating aquaculture systems (RAS) (Bergheim *et al.*, 2009; Ebeling and Timmons, 2012).

One concern to implementing RAS for salmon's FW development is that the environment provided differs from other culture environments, potentially impacting their performance once transferred to SW for grow-out. For example, it has been suggested that use of RAS affects smolts' development in FW, with physiological and molecular differences observed in these fish relative to those reared in alternative culture systems (Roque d'Orbcastel *et al.*, 2009; Kolarevic *et al.*, 2014; van Rijn *et al.*, 2020; Lai *et al.*, 2024). These differences have been thought to impact the osmoregulatory ability of RAS-reared smolts once transferred to SW environment. Alternatively, Lai *et al.* (2024) suggested that the relative stability of the RAS environment does not appropriately prepare smolts for the heterogeneous environment of SW cages. As a potential result of these factors, poorer phenotypic and growth performance in SW of RAS-reared fish has been observed. However, the above comparisons have only been made between RAS and flow-through systems, with no equivalent research into the relative performance of RAS- and loch-reared smolts. Furthermore, while the performance of Atlantic salmon reared in RAS throughout the production cycle, relative to those exposed to both RAS and sea-cage environments has been investigated (Correa *et al.*, 2018; Gonzalez, Gallardo-Hidalgo and Yáñez, 2022), the influence on the FW environment on later life performance in SW has not been thoroughly explored.

One approach to studying the impact of FW environment on SW performance is via the identification and quantification of genotype-environment interactions (GxE). GxE forms when the genotypic performance is environmentally sensitive, such that there is a re-ranking

of families across environments (Falconer, 1952; Falconer and Mackay, 1996; Mulder *et al.*, 2006; Sae-Lim *et al.*, 2016). This is often quantified as the genetic correlation between the same trait when measured in the same population but reared in distinct environments (r_{gxe}) (Mulder *et al.*, 2006). Importantly, where this value falls below 0.7, selective gains may not being realised across environments, and use of environment-specific breeding programs could be of benefit (Sae-Lim *et al.*, 2013, 2016).

While working in a commercial population, the impact of RAS-rearing relative to that in an ambient loch system on Atlantic salmon smolt phenotypic traits, as well as their genetic architecture and GxE, was previously investigated in Chapter 2. Results revealed that although smaller, RAS-reared smolts carried greater estimates of heritability and lower trait variance, providing valuable insights into how both genetic and environmental factors influence phenotypic traits during this FW phase. Additionally, while weak GxE were identified for length and condition factor, lower r_{gxe} reported for whole-body weight indicated this should be considered in a program of selective breeding. Following the same population at three-months post-SW transfer, this study aims to investigate if FW-rearing impacts SW performance. The objectives include (1) evaluating the impact of FW-origin on SW performance, (2) investigating the genetic architecture and GxE interactions underlying SW traits, (3) assess how well FW performance predicts SW outcomes through GxE quantification.

4.3. Materials and Methods

4.3.1. Ethics

Animal handling and collection in this study was carried out following the UK Animals (Scientific Procedures) Act 1986 Amended Regulations (SI2012/3039) and the work was approved by the University of Stirling Ethics Committee (Animal Welfare and Ethics Review Board; (AWERB202148713775). This project was also part of a larger BBSRC/NERC funded project, ROBUSTSMOLT, which also received ethical approval (Comparative RAS Study 2585).

4.3.2. Experimental design

This study continues from that presented in Chapter 2, please refer to this or Tollervey *et al.* (2024) for more detailed information on the study population and freshwater (FW) rearing. In brief, the study population originated from the broodstock of the MOWI Ireland 2022 Generation. Approximately 250,000 eyed eggs were co-reared in RAS in the northwest of Scotland (Inchmore) for almost nine-months from January to September 2021. During this time parr underwent re-distribution amongst tanks several times and ~10% bottom cull. After this, the population was phenotypically sexed using ultrasound. After sexing, approximately half (91,708 fish) of the remaining population was transferred to floating net-pens in a FW loch (Loch Garry) for smoltification, with other half (91,713 fish) remaining in the RAS facilities. Phenotypically male and female were separately reared in both environments. In the loch environment, each was reared in a single net-pen, whereas in RAS phenotypic sexes was split across three tanks each. Fish were stocked at ~306 and ~22 fish/m³ in RAS and loch respectively. Both populations were fed MOWI Proetus diets. In RAS, parr were initially held under 12hr light/darkness (LND) cycles before exposure to continuous light for approximately one month. In the loch, parr were exposed to winter (without light) photoperiod before usual 400 degree days of light prior leading up to SW transfer.

After about eight weeks in their FW environments (from September to November 2021), both populations – RAS and loch pen - were transferred to the shared SW site for grow-out (Loch Torridon). Due to biomass limitations at the SW site, only fish from phenotypically female tanks/pen in the RAS and loch populations could be maintained separately. Specifically, the phenotypically male fish from both FW environments were transferred and pooled into the same cage. On the same day, the female pen from the loch environment was transferred to a single cage. The following week, all three female tanks from the RAS site were pooled and transferred to a single cage. The three populations of fish were housed in SW cages, each 120m wide, 16m deep, and 18,000m³ in volume. In total, 44,908 females were transferred from RAS, and 31,384 from loch environment, corresponding to a density of ~2.49 and ~1.74 fish/m³ for RAS and loch populations at the sea-cage site, respectively.

Fish in SW were exposed to the same husbandry conditions, were initially fed NEPTUNE 50 adapt (from SW transfer for approximately 50 days) before transfer to NEPTUNE 200 adapt

diet; and underwent a sea lice treatment using BioMar feed (Medi Adapt smolt LR 75, 3 mm pellet). The number of mortalities or moribund fish removed from each pen was recorded from approximately the day of transfer until the onset of SW sampling by MOWI site staff. Biomass limitation required the female population to be pooled after SW sampling. Measurements of temperature (°C), pH, and oxygen saturation (%) were taken in both FW environments, with additional water quality parameters recorded in the RAS environment (Figure 2-1, Appendix A. 2). Post-SW transfer, temperature (°C), salinity (‰), and water clarity were recorded daily. For each, the average and standard deviation (s.d) was taken across the study period.

4.3.3. Sample collection

The population was sampled twice, at the end of FW-rearing as smolts (November 2021), and approximately three-months post-SW transfer as post-smolts (March 2022). At both, 1,000 fish per FW-rearing environment (RAS and loch) were sampled. Sampling occurred by random netting of fish from tanks/pens in FW and cages at SW. In the latter fish were attracted to the surface for sampling with feed. At both, measurements of whole-body weight (WBW) and length (tip of the head (snout) to the deepest point of the fork in the caudal fin) (cm) were taken. Fin clips were taken from adipose fin and stored in 2ml 95% ethanol.

4.3.4. Genotyping, pedigree reconstruction, and population structure

Parental broodstock was previously genotyped to ~55K SNP (non-public Axiom array, 97 NOFSAL03) by MOWI collaborators. FW and SW offspring were genotyped to ~65K SNP by IdentiGEN Ltd (non-public Axiom array, SALMOWI). SNPs were called based on major allele frequency with Applied Biosystems – Analysis Power Tools (APT) v2.11.6. Genotypic sex was assigned based on sex-determining region Y gene expression. Pedigree reconstruction and family assignment were performed by MOWI using the sequenced genotypes and their in-house software which employs an opposite homozygosity (OH) method (additional details can be seen in Chapter 2.3.6). Fish which failed to be assigned to sire, dam, or both were removed.

Parental and FW-SW offspring (combined samples from FW and SW) separately underwent quality control (QC) using PLINK v1.9 (Purcell *et al.*, 2007) as in Chapter 2.3.5. A list of

common SNPs to both the parental and FW-SW offspring populations was then gathered from the overlap in the online Venn Diagrams resource at BioTools.Fr (<https://www.biotoools.fr/misc/venny>). This list was used to merge the two populations in PLINK v1.9 (Purcell *et al.*, 2007), which was formatted into .txt file using *genio* package (Ochoa, 2023) in R v4.2.2 (R Core Team, 2022). The genomic relationship matrix (GRM) between all individuals was computed using GCTA software (Yang *et al.*, 2011). The first two components of PCA on the GRM were visualised separately for FW and SW offspring using *ggplot* package (v3.4.2) in R v4.2.2 (R Core Team, 2022) to assess the presence of any population structure. The number of full and half-sib (sire and dam families) were investigated per location (FW and SW) and FW-origin (RAS and loch), with only those common across all four populations (i.e. FW-RAS, FW-Loch, SW-RAS and SW-Loch) retained for analysis.

4.3.5. Phenotypic traits

Of the fish sampled at the FW stage, only those in pens maintained separately by FW-origin once transferred to SW were retained for further analysis. Samples with missing phenotypic data were removed. The FW whole-body weight (WBW_{FW}) and length (length_{FW}), and SW whole-body weight (WBW_{SW}) and length (length_{SW}) were recorded. Condition factor (K), was also calculated as $K = 100 * (W_{\text{grams}}/L_{\text{cm}}^3)$ following Fulton, (1904).

Additionally, for the SW population, average daily gain (ADG), representing a change in trait value per day (Δ/day) was calculated as $(X_F - X_I)/\text{days in SW}$ where, X_F is the final trait value measured in SW and X_I is the initial trait value measured at the end of the FW-rearing period (Crane, Ogle and Shoup, 2020). Also, specific growth rate (SGR) was calculated as $100 * e^{g-1}$ where $g = (\log(X_F) - \log(X_I))/\text{days in SW}$, expressed as percent change in trait value per day ($\%\Delta/\text{day}$), with X_F and X_I as above (Crane, Ogle and Shoup, 2020). ADG and SGR were calculated for both WBW (ADG_{WBW} and SGR_{LENGTH}) and length (SGR_{WBW} and ADG_{LENGTH}). Of note, as fish were not tagged and tracked throughout the study, the initial trait values (X_I) were based on the average family value measured at the end of FW-rearing (i.e. Chapter 2), calculated separately for the RAS and loch environment. To allow methodological differences to be accounted for in statistical analysis, 'days in SW', the number of days between the date of SW transfer and SW sampling, as well as 'extra FW days', the number of days between the

date of FW sampling and SW transfer (days since FW sampling – days in SW), were recorded (Appendix C. 1).

4.3.6. Statistical analysis

All statistics were performed in R v4.2.2 (R Core Team, 2022). Mean and standard deviation (s.d) were calculated for each trait in the overall SW and FW populations, as well as by FW environment. The effect of FW environment, genotypic sex, and interaction between FW environment and sex was investigated through 2-way-ANOVA (Type II sequential) with Post Hoc Tukey test (P-value < 0.05) on the FW and SW populations separately. Validity of test assumptions was assessed visually via plotting a histogram as well as post-statistical testing q-q plots. Where test assumptions were invalid, non-parametric Kruskal–Wallis test was performed to separately assess the effect of FW environment and genotypic sex (P-value < 0.05).

4.3.7. Quantitative analysis

All analysis was performed using BLUPF90 (Miszta *et al.*, 2002) software.

4.3.7.1. Within environment genetic parameters

For both FW and SW populations, genetic parameters of traits were estimated separately for the loch and RAS environment from univariate animal mixed models (Eq.4-1) in which variance components were estimated via the use of a restricted maximum likelihood (REML) approach.

$$\mathbf{y} = \boldsymbol{\mu} + \mathbf{X}\mathbf{b} + \mathbf{Z}\mathbf{u} + \mathbf{e} \quad \text{Eq. 4-1}$$

In this, $\mathbf{y}$ was a vector of trait values, $\boldsymbol{\mu}$ the overall mean, $\mathbf{X}$ a design matrix linking individuals to a vector of fixed effects, $\mathbf{b}$. $\mathbf{Z}$ was a design matrix linking individuals to a vector of additive genetic effects $\mathbf{u}$, fitted using single-step genomic evaluation. In this, $\mathbf{u}$ has a multivariate normal distribution (MVN) with mean zero and variance $V_g * \mathbf{H}$ where V_g is additive genetic variance and $\mathbf{H}$ is a matrix that combines information from both pedigree ($\mathbf{A}$ matrix) and SNP data ($\mathbf{GRM}$), as defined in Legarra *et al.* (2014) ($\mathbf{u} \sim \text{MVN}(0, V_g * \mathbf{H})$). Lastly, $\mathbf{e}$ was a vector of residual effects with $\sim \text{MVN}(0, V_r * \mathbf{I})$, where $\mathbf{I}$ was the identity matrix and V_r is residual

variance. Full pedigree and SNP data were provided to each model but with only phenotypic data from one of the four populations.

Depending on the population and trait of interest, fixed effects changed. For traits measured at the FW stage, only genotypic sex (2 levels; 1=male, 2=female) was included. For the SW-RAS population growth traits genotypic sex (2 levels, 1=male, 2=female), SW days (3 levels, Appendix C. 1) and extra FW days (2 levels, Appendix C. 1) were also included. For SW fish of loch-origin, extra FW was omitted as only a single level existed within this population (Appendix C. 1). For SW phenotypic traits (WBW_{SW} , $length_{SW}$, and K_{SW}) covariate of the initial FW trait value was also included. Specifically, average family WBW measured at the end of FW-rearing was included for WBW_{SW} and K_{SW} , and the average family length measured at the end of FW-rearing for $length_{SW}$.

For each trait, narrow sense heritability ($h^2 = V_g/V_p$), coefficient of phenotypic variance ($CV = (SD_p/\bar{X}) * 100$, where $\bar{X}$ is the phenotypic mean) and coefficient of genetic variance ($CGV = (\sqrt{V_g}/\bar{X}) * 100$) was estimated (Falconer and Mackay. 1996; Cheung, 2020).

4.3.7.2. Genotype-environment interactions

GxE was estimated using a multi-trait approach in which the same trait measured in different environments was treated as two independent traits (Mulder and Bijma, 2005; Sae-Lim *et al.*, 2014). This was implemented by use of bi-variant animal mixed models (Eq. 4-2), with variance components estimated via the use of a restricted maximum likelihood (REML) approach.

$$\begin{pmatrix} y_1 \\ y_2 \end{pmatrix} = \begin{pmatrix} x_1 & 0 \\ 0 & x_2 \end{pmatrix} \begin{pmatrix} b_1 \\ b_2 \end{pmatrix} + \begin{pmatrix} z_1 & 0 \\ 0 & z_2 \end{pmatrix} \begin{pmatrix} u_1 \\ u_2 \end{pmatrix} + \begin{pmatrix} e_1 \\ e_2 \end{pmatrix} \quad \text{Eq. 4-2}$$

In this, y_1 and y_2 are the same trait in environment one and environment two, respectively. Random and fixed effects followed that of the univariate analysis. Residual covariance was set to zero (Sae-Lim *et al.*, 2016). The strength of GxE was quantified by the genetic correlation (r_{gxe}) between the two traits (Mulder and Bijma, 2005; Sae-Lim *et al.*, 2014). Specifically, this approach was used to estimate r_{gxe} between (1) the same trait measured in each environment (RAS and loch) at the end of the FW-rearing period (FW GxE), (2) the same trait measured in each environment (RAS and loch) but in the SW population (SW GxE), and (3) between the

same trait measured in the same environment (either RAS or loch) but between FW and SW populations (FW-SW GxE) (Figure 4-1).

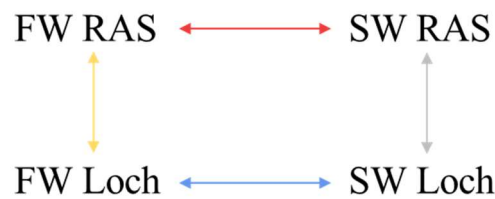


Figure 4-1 GxE comparison made where yellow is FW GxE, grey is SW GxE between RAS and loch populations. Blue and red are FW-SW GxE for the loch and RAS populations, respectively.

4.4.Results

4.4.1. Saltwater environmental description

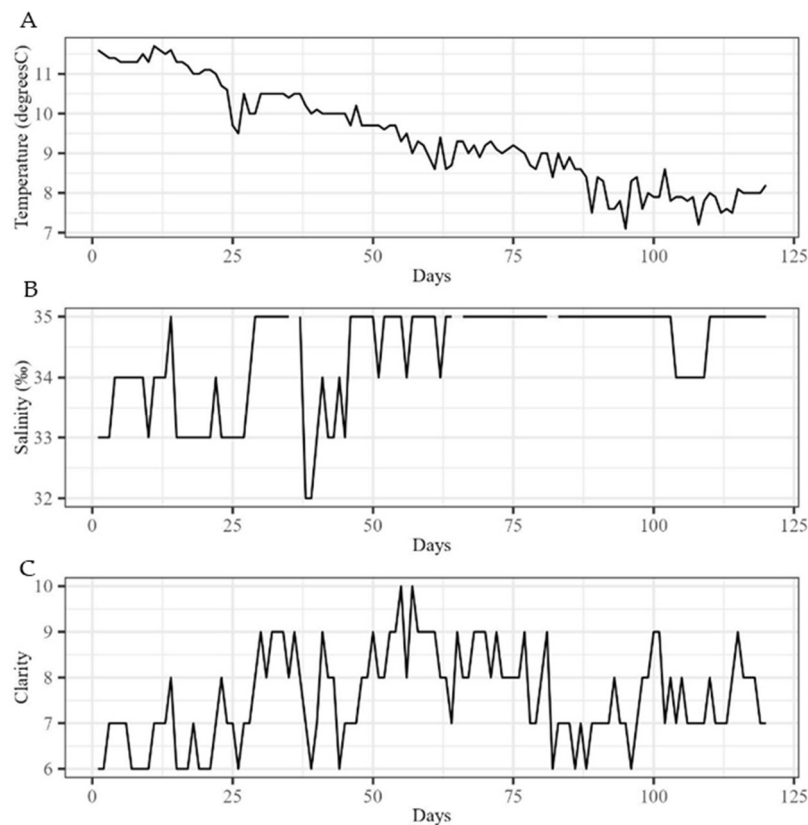


Figure 4-2 A) Temperature (°C), B) Salinity (‰) and C) clarity measurements for the first 120 days post-SW transfer stage.

Environmental data of the post-SW transfer stage is shown in Figure 4-2. The average SW temperature was 9.41°C (s.d 1.26), clarity 7.55 (s.d 1.03) and salinity 34.42 ‰ (s.d 0.83). While salinity and clarity fluctuate around the mean, temperature decreased gradually with time from approximately 12°C to 8°C (Figure 4-2).

4.4.2. Genotyping, quality control and population description

In FW, mortality rates were low, 2.90% and 4.18% for FW-Loch and FW-RAS populations respectively. After transfer to SW, mortality was lower, at 0.32% for SW-RAS and 0.37% for SW-Loch populations. A total of 4,000 fish were sampled in combined FW-SW offspring. After sequencing, genotypic information was available for a total of 3,919 individuals. In the parental population 254 individuals were genotyped. Raw parental and FW-SW offspring genotypes contained 55,357 SNPs, and 65,7745 SNPs, respectively, with 53,157 and 53,788 remaining after QC (Appendix C. 2). There were 41,127 SNPs common to both (Appendix C. 3). Based on filtered SNPs and GRM, there was little indication of population structure, with results from FW and SW populations highly similar (Appendix C. 4).

Table 4-1 The number of fish of each genotypic sex and freshwater-rearing environment (RAS or loch) for both freshwater (FW) and saltwater (SW) populations.

	FW			SW		
	RAS	Loch	Total	RAS	Loch	Total
<i>Male</i>	432	172	604	324	262	586
<i>Female</i>	234	478	712	586	622	1208
<i>Total</i>	666	650	1,316	910	884	1,794

After QC, only FW smolts belonging to tanks which were separately maintained by FW-origin post-transfer were used in further analysis. This left 3,429 in the FW-SW offspring. Pedigree re-construction identified 90 sire-, 163 dam-, and 166 full-sib families. For 37 samples, neither a sire nor a dam could be assigned, with a further 28 not assigned to a sire only. Across FW-RAS, FW-Loch, SW-RAS and SW-Loch populations, 124 full-sib families were shared. A full categorical breakdown of this remaining population, a total of 3,110 smolts and post-smolts, can be seen in Table 4-1. Of FW smolts, both average family size and its variance were similar between the RAS (5.24, s.d 2.73) and loch (5.24, s.d 2.94) populations. These values were approximately two fish smaller than the family size of sampled post-smolts. Specifically, average family size and variance of the SW population were similar between RAS (7.34, s.d 3.92) and loch (7.13, s.d 4.17) origin families. Across all four populations total family size was highly variable, ranging from 5 to 47.

4.4.3. Phenotypic parameters

All traits approximated normality visually, except for the condition factor in the SW population (Appendices C. 5-C. 9).

4.4.3.1. Freshwater smolts

Table 4-2 Phenotypic and genetic parameters of smolts sampled at the end of freshwater-rearing, where WBW is whole-body weight, K is condition factor, CV% is coefficient of phenotypic variance, CGV% is coefficient of genetic variance, V_p is phenotypic variance, V_g is additive genetic variance, V_r is residual variance, h^2 is narrow sense heritability, s.d the standard deviation, and s.e is standard error.

	WBW _{FW} (g)		Length _{FW} (cm)		K _{FW}	
	RAS	Loch	RAS	Loch	RAS	Loch
<i>Mean (s.d)</i>	85.22 (14.78)	120.81 (26.82)	18.78 (1.02)	21.24 (1.68)	1.28 (8.07e ⁻²)	1.25 (1.00e ⁻¹)
<i>CV%</i>	17.35%	22.20%	5.44%	7.92%	6.28%	8.04%
<i>V_p (s.e)</i>	223.37 (14.20)	740.55 (42.97)	1.06 (0.65e ⁻¹)	2.92 (0.17)	0.62e ⁻² (0.39e ⁻²)	0.12e ⁻¹ (0.67e ⁻³)
<i>V_g (s.e)</i>	72.97 (18.28)	130.71 (43.91)	0.27 (0.79e ⁻¹)	0.41 (0.16)	0.24e ⁻² (0.48e ⁻³)	0.12e ⁻² (0.62e ⁻³)
<i>V_r (s.e)</i>	150.48 (13.87)	610.00 (446.124)	0.79 (0.67e ⁻¹)	2.51 (0.19)	0.38e ⁻² (0.35e ⁻³)	0.11e ⁻¹ (0.78e ⁻³)
<i>CGV%</i>	10.02%	9.46%	2.77%	3.01%	3.83%	2.77%
<i>h² (s.e)</i>	0.32 (0.71e ⁻¹)	0.18 (0.56e ⁻²)	0.25 (0.55e ⁻¹)	0.14 (0.54e ⁻¹)	0.38 (0.64e ⁻¹)	0.10 (0.51e ⁻¹)

Phenotypic and genetic parameters of FW smolts can be seen in Table 4-2. On average, WBW_{FW}, length_{FW}, and K_{FW} were 103.35g (s.d 28.41g), 37.97cm (s.d 2.04cm), and 1.41 (s.d 0.13) respectively, and carried a CV of 27.49%, 5.37%, and 9.33%. Loch-reared smolts were significantly heavier (degrees of freedom=1/1312, F-value=809.00, P-value<2e⁻¹⁶) and longer (degrees of freedom=1/1312, F-value=946.53, P-value<2e⁻¹⁶) than their RAS counterparts. However, RAS smolts displayed a greater condition factor than those from the loch environment (degrees of freedom=1/1312, F-value=30.35, P-value=4.33e⁻⁸) (Table 4-2, Appendix C.5). Neither sex (length_{FW}: degrees of freedom=1/1312, F-value=3.39, P-value=0.066, K_{FW}: degrees of freedom=1/1312, F-value=0.33, P-value=0.57) nor sex-by-environment interaction (length_{FW}: degrees of freedom=1/1312, F-value=0.74, P-value=0.39, K_{FW}: degrees of freedom=1/1312, F-value=2.91, P-value=0.089) had a significant effect on length_{FW} and K_{FW} (Figure 4-3). In contrast sex (degrees of freedom=1/1312, F-value=4.07, P-value=0.044) but not sex-by-environment interaction (degrees of freedom=1/1312, F-value=2.34, P-value=0.13) significantly affected WBW_{FW} (Figure 4-3). Specifically, females were on average 2.30g (s.d 2.18g) lighter than their male counterparts. Across traits, estimates of variance were lower in the RAS compared to those of loch population (Table 4-2).

4.4.3.2. Saltwater post-smolts

Phenotypic and genetic parameters of SW post-smolts sampled can be seen in Table 4-3. On average SW post-smolts were 781.97g (s.d 152.58g) in weight (WBW_{SW}) and 37.97cm (s.d 2.04cm) in length ($length_{SW}$), with a condition factor (K_{SW}) of 1.41 (s.d 0.13). The coefficient of variation (CV%) was 19.51%, 5.37%, and 9.33%, for WBW_{SW} , $length_{SW}$, and K_{SW} , respectively. Fish of loch-origin were significantly heavier (degrees of freedom=1/1790, F-value=162.57, P-value<2e⁻¹⁶) and longer (degrees of freedom=1/1790, F-value=204.45, P-value<2e⁻¹⁶) than RAS counterparts (Table 4-3, Appendix C. 6).

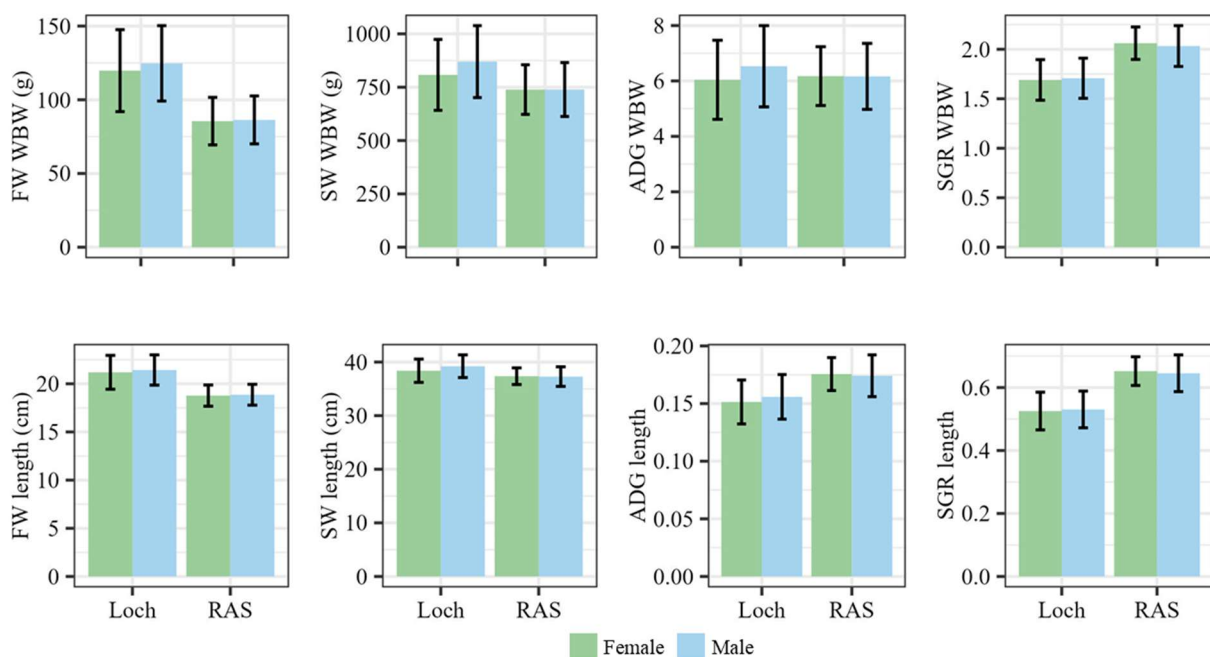


Figure 4-3 Mean and standard deviation (s.d) for freshwater and saltwater traits, split by freshwater environment (RAS and loch) and genotypic sex, where green are females and blue are males.

Sex had a significant effect on weight (WBW_{SW} : degrees of freedom=1/1790, F-value=16.07 P-value=6.36e⁻⁵) and length ($length_{SW}$: degrees of freedom=1/1790, F-value=13.25, P-value=2.81e⁻⁴) in saltwater. Specifically, female fish were on average 29.19g (s.d 14.31g) lighter and 0.35cm (s.d 0.19) shorter than males. Additionally, sex by FW environment interaction also significantly affected these traits (WBW_{SW} : degrees of freedom=1/1790, F-value=17.87, P-value=2.48e⁻⁴, $length_{SW}$: degrees of freedom=1/1790, F-value=22.38, P-value=2.42e⁻⁶). Where there was a significant difference between the sexes when measured in the loch environment only (Figure 4-3). Neither FW environment (degrees of freedom=1, Chi-squared=2.57, P-value=0.11) nor sex (degrees of freedom=1, Chi-squared=3.21 P-value=0.07) had a significant

effect on K_{SW} . Phenotypic traits, except for K_{SW} , were less variable in RAS-reared post-smolts (Table 4-3).

Table 4-3 Phenotypic and genetic parameters of post-smolts sampled three-months post-saltwater transfer, where WBW is whole-body weight, K is condition factor, CV% is coefficient of phenotypic variance, CGV% is coefficient of genetic variance, V_p is phenotypic variance, V_g is additive genetic variance, V_r is residual variance, h^2 is narrow sense heritability, s.d the standard deviation, and s.e standard error.

	WBW _{SW} (g)		Length _{SW} (cm)		K_{SW}	
	RAS	Loch	RAS	Loch	RAS	Loch
<i>Mean (s.d)</i>	738.9 (119.82)	826.25 (169.19)	37.33 (1.65)	38.63 (2.19)	1.41 (0.14)	1.41 (0.13)
<i>CV%</i>	16.21%	20.48%	4.42%	5.66%	9.76%	8.86%
<i>V_p (s.e)</i>	14061 (740.30)	26952 (1513)	2.61 (0.13)	4.52 (0.25)	$0.19e^{-1}$ ($0.93e^{-3}$)	$0.15e^{-1}$ ($0.81e^{-3}$)
<i>V_g (s.e)</i>	3428.3 (859.44)	9227.7 (1803.6)	0.53 (0.14)	1.57 (0.30)	$0.30e^{-2}$ ($0.95e^{-3}$)	$0.37e^{-2}$ ($0.89e^{-3}$)
<i>V_r (s.e)</i>	10636 (720.63)	17734 (1286.0)	2.08 (0.13)	2.95 (0.22)	$0.16e^{-1}$ ($0.97e^{-3}$)	$0.12e^{-1}$ ($0.77e^{-3}$)
<i>CGV%</i>	7.93%	11.62%	1.95%	3.37%	3.88%	4.31%
<i>h^2 (s.e)</i>	0.24 ($0.55e^{-1}$)	0.34 ($0.56e^{-1}$)	0.20 ($0.50e^{-1}$)	0.35 ($0.56e^{-1}$)	0.16 ($0.48e^{-1}$)	0.24 ($0.51e^{-1}$)

Estimates of post-smolt growth rate over the first three-months in SW, as well as their genetic parameters, can be seen in Table 4-4. On average, growth rate was 1.88 g/day (s.d 0.26 g/day), 0.59 cm/day (s.d $8.25e^{-2}$ cm/day), 6.18 %g/day (1.29 %g/day) and 0.16 %cm/day (s.d $2.08e^{-2}$ %cm/day) for ADG_{WBW}, ADG_{LENGTH}, SGR_{WBW}, and SGR_{LENGTH}, respectively. The coefficient of variance (CV%) was 13.98%, 14.01%, 20.88%, and 12.68% for ADG_{WBW}, ADG_{LENGTH}, SGR_{WBW}, and SGR_{LENGTH} respectively (Table 4-4). ADG_{WBW} was not significantly affected by FW-origin (degrees of freedom=1/1790, F-value=0.066, p=0.80) (Appendix C. 7). However, both sex (degrees of freedom=1/1790, F-value=12.02, p= $5.38e^{-4}$) and sex-by-environment interaction (degrees of freedom=1/1790, F-value=14.79, p= $1.24e^{-4}$) had a significant impact on this trait. Specifically, in males WBW was increasing at a faster rate than females, with a difference in sex performance only seen in those of loch origin (Figure 4-3).

FW-origin had a significant impact on ADG_{LENGTH} (degrees of freedom=1/1790, F-value=731.00, P-value< $2e^{-16}$), SGR_{WBW} (degrees of freedom=1/1790, F-value=1533.59, P-value< $2e^{-16}$), and SGR_{LENGTH} (degrees of freedom=1/1790, F-value=2231.47, P-value< $2e^{-16}$) where fish of RAS-origin appeared to be growing at an increased rate compared to loch counterparts (Table 4-4 and Appendix C. 7). Sex was not found to significantly affect ADG_{LENGTH} (degrees of freedom=1/1790, F-value=2.17, P-value=0.14), SGR_{WBW} (degrees of freedom=1/1790, F-value=0.55, P-value=0.46) and SGR_{WBW} (degrees of freedom=1/1790, F-value=0.20, P-value=0.66), although, sex-by-environment FW-origin did (ADG_{LENGTH}: degrees of

freedom=1/1790, F-value=11.14, P-value=8.62e⁻⁴; SGR_{WBW}: degrees of freedom=1/1790, F-value=5.50, P-value=1.92e⁻²; SGR_{WBW}: degrees of freedom=1/1790, F-value=4.50, P-value=3.41e⁻²) (Figure 4-3). Specifically, in post-smolts of loch-origin, males appeared to be growing at a faster rate than females (Figure 4-3). These within environment differences was significant for ADG_{LENGTH}, but not SGR_{WBW} and SGR_{LENGTH}. Across traits, estimates were less variable in RAS-origin post-smolts (Table 4-4).

Table 4-4 Phenotypic and genetic parameters of post-smolts sampled three-months post-saltwater transfer, where ADG is average daily gain, SGR is specific growth rate, CV% is coefficient of phenetic variance, CGV% is coefficient of genetic variance, V_p is phenotypic variance, V_g is additive genetic variance, V_r is residual variance, h^2 is narrow sense heritability, s.d the standard deviation and s.e the standard error

	ADG _{WBW} (g/day)		ADG _{LENGTH} (cm/day)		SGR _{WBW} (%g/day)		SGR _{LENGTH} (%cm/day)	
	RAS	Loch	RAS	Loch	RAS	Loch	RAS	Loch
<i>Mean (s.d)</i>	6.17 (1.11)	6.19 (1.45)	0.18 (1.58e ⁻²)	0.15 (1.92e ⁻²)	2.05 (0.18)	1.70 (0.20)	0.65 (5.05e ⁻²)	0.53 (5.95e ⁻²)
<i>CV%</i>	17.96%	23.51%	9.00%	12.60%	8.80%	12.06%	7.78%	11.92%
<i>V_p (s.e)</i>	1.25 (0.65e ⁻¹)	2.08 (0.12)	0.25e ⁻³ (0.13e ⁻⁴)	0.38e ⁻³ (0.22e ⁻⁴)	0.34e ⁻¹ (0.20e ⁻²)	0.42e ⁻¹ (0.26e ⁻²)	0.26e ⁻² (0.16e ⁻³)	0.35e ⁻² (0.22e ⁻³)
<i>V_g (s.e)</i>	0.29 (0.75e ⁻¹)	0.72 (0.14)	0.62e ⁻⁴ (0.14e ⁻⁴)	0.16e ⁻³ (0.22e ⁻⁴)	0.16e ⁻¹ (0.26e ⁻²)	0.22e ⁻¹ (0.32e ⁻²)	0.12e ⁻² (0.20e ⁻³)	0.21e ⁻² (0.27e ⁻³)
<i>V_r (s.e)</i>	0.97 (0.64e ⁻¹)	1.36 (0.98e ⁻¹)	0.19e ⁻³ (0.13e ⁻⁴)	0.22e ⁻³ (0.17e ⁻⁴)	0.18e ⁻¹ (0.15e ⁻²)	0.21e ⁻¹ (0.17e ⁻²)	0.13e ⁻² (0.11e ⁻³)	0.14e ⁻² (0.13e ⁻³)
<i>CGV%</i>	8.73%	13.71%	4.37%	8.43%	6.17%	8.72%	5.33%	8.65%
<i>h² (s.e)</i>	0.23 (0.54e ⁻¹)	0.35 (0.56e ⁻¹)	0.24 (0.50e ⁻¹)	0.42 (0.55e ⁻¹)	0.46 (0.57e ⁻¹)	0.51 (0.52e ⁻¹)	0.47 (0.55e ⁻¹)	0.59 (0.49e ⁻¹)

4.4.4. Genetic variance and trait heritability

For the H matrix, mean allele frequency was 0.28, with 0.40% of genotypes missing. The correlation between GRM and A matrix diagonal elements was 0.42, marginally below the 0.5 threshold. The correlation of the off-diagonal elements was greater at 0.71.

For all FW traits, estimates of residual and phenotypic variance were consistently greater in the loch-reared compared to RAS-reared population (Table 4-2). While additive genetic variance was similar between environments for length_{FW} and WBW_{FW}, for K_{FW} this was greater in the RAS population. In turn, the estimated heritabilities were consistently greater in the RAS population. Specifically, these were moderate in the RAS (0.25-0.38) and low-to-moderate in the loch (0.10-0.18). Across SW traits, heritability estimates demonstrated a wide range (Table 4-3 and Table 4-4). For all SW traits, estimates of phenotypic and residual variance were greater in the loch- than RAS-origin fish. This was at the exception of condition factor. Estimates of additive genetic variance were also greater in the loch population. This translated into consistently greater heritability estimates in the loch-origin population.

4.4.5. Genotype-Environment interactions

4.4.5.1. FW GxE

The genetic correlations (r_{gxe}) suggest moderate-to-weak GxE effects for FW traits, with the strongest effect observed for WBW_{FW} (0.49, s.e 0.23) followed by $length_{FW}$ (0.73, s.e 0.33), and condition factor (K_{FW}) (1.02, s.e 0.67). It should be noted that heritabilities carried high error estimates (in particular, for condition factor).

4.4.5.2. SW GxE

Genetic correlations indicate weak GxE for phenotypic traits (Table 4-5). For growth traits, results suggest moderate-to-strong GxE effects at the exception of ADG_{WBW} , where a high genetic correlation was recorded. Of note, r_{gxe} indicate GxE were stronger for length compared to WBW based growth rates.

Table 4-5 Genetic correlations (r_{gxe}) and standard error (s.e) for saltwater (SW) phenotypic (WBW_{SW} , $length_{SW}$, and K_{SW}) and growth (ADG_{WBW} , ADG_{LENGTH} , SGR_{WBW} , and SGR_{LENGTH}) traits. WBW is whole-body weight, K is condition factor, ADG is average daily gain, and SGR is specific growth rate

	WBW_{SW}	$Length_{SW}$	K_{SW}	ADG_{WBW}	ADG_{LENGTH}	SGR_{WBW}	SGR_{LENGTH}
r_{gxe}	0.86	0.97	0.93	0.87	0.6	0.21	0.17
<i>s.e</i>	0.10	9.00e ⁻²	0.15	9.90e ⁻²	0.13	0.11	0.10

4.4.5.3. FW-SW GxE

Genetic correlation between phenotypic traits measured at the end of FW-rearing and then three-months post-SW transfer indicate moderate-to-weak GxE (Table 4-6). Results indicate similar GxE effects when compared between RAS and loch populations. This is at the exception of estimate for WBW in the loch population which was lower than that of the RAS-reared fish. Error estimates were consistently greater for r_{gxe} in the loch population.

Table 4-6 Genetic correlation (r_{gxe}) and standard error (s.e) for phenotypic traits whole-body weight (WBW) (g) length (cm) and condition factor (K) for the RAS- and loch-reared populations measured as smolts at the end of freshwater-rearing and as post-smolts approximately three-months post-saltwater transfer.

	WBW (s.e)	Length (s.e)	K (s.e)
RAS	0.82 (0.11)	0.82 (0.13)	0.78 (0.15)
Loch	0.57 (0.24)	0.78 (0.34)	0.85 (0.49)

4.5. Discussion

While the freshwater (FW) husbandry environment employed within the UK for Atlantic salmon production is predominantly net-pens in lochs, over recent years there has been increased adoption of land-based systems, such as the fully-closed recirculating aquaculture systems (RAS) (Bergheim *et al.*, 2009; Clarke and Bostock, 2017). However, the difference in environmental conditions between these two culture systems may have implications for the development and performance of commercial stocks, with potential consequence for longer term growth in saltwater (SW). One way to investigate this is via genotype-environment interactions (GxE) which occur when family performance shows inconsistency between environments (Falconer and Mackay, 1996; Mulder and Bijma, 2005).

Working within a commercial setting, the presence of GxE between a population separately reared in either a RAS or ambient loch system for approximately eight weeks leading up to smoltification was previously investigated in Chapter 2. Results indicated that while RAS-rearing produced smolts that were smaller in terms WBW and length, they carried greater estimates of heritability and condition factor. Furthermore, while only weak GxE were identified for length and condition factor, moderate estimates for WBW suggest that their consideration in future breeding decisions could help improve selective gains (Mulder *et al.*, 2006; Sae-Lim *et al.*, 2016). Although the specific environmental factors driving this result could not be identified, RAS specific variables that differ from the loch environment (i.e. temperature, stocking density or water quality) were suggested as a possible cause. Here, similar analyses were performed at the end of FW-rearing, but on a reduced data set, with results in agreement with those presented in Chapter 2 (Table 4-2).

While it is important to note there is no 'standard' RAS environment, these results contrast with previous reports which did not observe phenotypic differences in RAS-reared smolts at the end of FW-rearing (Kolarevic *et al.*, 2014; van Rijn *et al.*, 2020; Lai *et al.*, 2024). However, these latter studies compared smolt performance in RAS to that of flow-through systems rather than ambient loch. As both RAS and flow-through systems are land-based with, at least partial, water recirculation, they may be more alike than RAS and loch in terms of environmental parameters. Furthermore, Lai *et al.* (2024) directly controlled environmental conditions to reduce the difference seen between FW culture systems compared.

Consequently, it may be that the greater degree of difference between RAS and ambient loch system drove the observed phenotypic difference and moderate the GxE for weight. Similarly, comparatively weaker GxE have been estimated between systems which are more similar than those that strongly differ (Sae-Lim *et al.*, 2013; Turra *et al.*, 2016).

4.5.1. SW phenotypic and growth performance

In this chapter, the same population as Chapter 2 was studied, but at three-months after the RAS and loch populations were transferred into the same SW environment for grow-out. The results presented here showed that compared to the end of FW-rearing (discussed in Chapter 2), at this stage, loch-origin fish were still larger in terms of whole-body-weight (WBW_{SW}) and length (length_{SW}), but now with no difference in condition factor (K_{SW}) (Table 4-3). Previous studies have demonstrated that early SW performance is affected by the smolt status prior to transfer, which itself can be shaped by the specific FW-rearing environment. For example, underpinning molecular and physiological differences have been reported at this stage of development, which has then been related to poorer osmoregulation post-SW transfer in RAS-reared fish (Kolarevic *et al.*, 2014; van Rijn *et al.*, 2020; Lai *et al.*, 2024). One marker of smoltification state is condition factor. During this process, condition factor typically decreases due to the elongation of body form (McCormick *et al.*, 1998; McCormick, 2012). The lower condition factor reported in the FW-Loch population may therefore indicate a greater state of preparedness for SW transfer, in turn enabling better SW performance. It is worth noting, however, that many other factors, some of which were recorded as differing at the FW stage (Appendix A. 2 and Figure 2-1), are known to affect condition factor and could have contributed to the difference seen here.

In contrast to these results, and despite their smaller size, RAS-origin post-smolts appeared to be growing at a significantly greater rate (Table 4-4). This may be indicative of compensatory growth, where in the FW RAS environment growth was limited by a lower carrying capacity in terms of total biomass, which once removed on SW transfer allowed growth to improve (Ellis *et al.*, 2002). Similarly, indications of compensatory growth in salmon have previously been suggested and linked to specific environmental factors such as food deprivation (Stefansson, Imsland and Handeland, 2009; Hvas *et al.*, 2022) or density changes (Refstie and Kittelsen, 1976). This suggests that RAS smolts may be smaller after three-months of SW

growth due to their smaller size at transfer, rather than a difference in smoltification state. Given additional time in SW, these fish may have been able to ‘catch-up’ to those reared in the FW loch environment.

While this result contrasts with previous studies (Kolarevic *et al.*, 2014; van Rijn *et al.*, 2020; Lai *et al.*, 2024), it does reiterate that early environments can lay the basis of performance in later life (Jonsson and Jonsson, 2014). Furthermore, Lai *et al.* (2024) demonstrated that the differences established in SW performance in the initial months post-transfer due to the use of specific FW environments created divergent growth trajectories that continued to harvest. Therefore, the enhanced growth performance of RAS smolts may have longer-term implications for their growth and performance throughout the whole production cycle. While it was not possible to separately rear RAS and loch populations beyond three-months post-transfer, measurements of growth through to harvest would be of interest to collect in the future to verify if the FW-rearing environment has an impact at more distal time periods.

4.5.2.Trait heterogeneity

It is worth noting that while variance estimates for SW traits aligns with those previously reported (Gjerde, Simianer and Refstie, 1994; Neira *et al.*, 2004; Quinton, McMillan and Glebe, 2005; Gonzalez, Gallardo-Hidalgo and Yáñez, 2022), those of the RAS-reared population were – except for condition factor – were lower than post-smolts of loch-origin (Table 4-3 and Table 4-4). This is in agreement with the results seen at the end of FW-rearing (Table 4-2), though in other studies observations have been inconsistent (Vandeputte *et al.*, 2010; Sae-Lim *et al.*, 2014; Li *et al.*, 2018; Van Sang *et al.*, 2020). Considering this, it has been suggested that the increased environmental control provided in RAS creates more stable husbandry environments which reduces trait heterogeneity (Vu *et al.*, 2021). While this may have been a possible driver of the decreased variance in FW, the results presented here demonstrated that this was maintained even after the RAS population entered the more varied SW environment (Figure 4-2).

Notably, sex and sex-by-environment interaction had a significant effect on some FW and SW traits. However, a difference in sex-specific performance was generally only observed in the loch population, where males were either larger or growing at faster rate than female counterparts (Figure 4-2). This is in agreement with previous studies (Gjerde, Simianer and

Refstie, 1994; Leclercq *et al.*, 2010; Thorland *et al.*, 2020). It is worth mentioning that growth rate is linked to maturation, where early maturing fish have been found associated with enhanced growth rates (Gjerde, Simianer and Refstie, 1994; Leclercq *et al.*, 2010). As male salmon can undergo maturation earlier than females (Klemetsen *et al.*, 2003; Good and Davidson, 2016), and because the onset of sexual maturation is dependent on achieving a certain body size or condition threshold (Thorpe, 1989; Thrope, 1994), given the evidence that the loch population achieved a greater size in FW (Figure 4-2), this environment and its specific conditions may have also favoured sexual dimorphism in growth. It is therefore unfortunate maturation was not assessed during this study

While elevated water temperature and exposure to continuous light have been linked to enhanced growth rates as well as higher proportion of early maturing males (Berrill *et al.*, 2003; Imsland, Handeland and Stefansson, 2014), such environmental conditions were more like the FW RAS rather than loch environment (Figure 2-1). However, the FW-Loch population were also reared at a lower density, which has additionally been seen to result in greater levels of early male maturation (McLay *et al.*, 1992; Berg *et al.*, 1996; Pino Martinez *et al.*, 2021). Therefore, just as the high density in RAS may have limited total biomass per tank during FW-rearing, the restriction this could have put on individual growth may have prevented the development of sexually dimorphic growth, as was observed in the FW-Loch population. In considering this finding, the difference in sex ratio between FW environments should, however, also be noted (Table 4-1). Specifically, in the FW-RAS population there was an excess of genotypic males relative to the loch environment. Despite this, these results indicate an overall loch specific pattern of enhanced male growth which may have contributed to the greater trait heterogeneity observed in this population, both pre- and post-SW transfer (Table 4-2 to Table 4-4). Such results could be worth further investigating.

4.5.3. Trait heritability and genetic variance

Heritability estimates for SW phenotypic traits (WBW_{SW}, length_{SW}, and K_{SW}) ranged from low-to-moderate (Table 4-3), falling within the range previously reported in salmonids (Neira *et al.*, 2004; Quinton, McMillan and Glebe, 2005; Powell *et al.*, 2008; Correa *et al.*, 2018; Thorland *et al.*, 2020; Gonzalez, Gallardo-Hidalgo and Yáñez, 2022). For growth rate measurements, reports on heritability are less available, with a wide range reported in teleost (~0.05-0.60)

(Dupont-nivet *et al.*, 2010; Vandeputte *et al.*, 2010; Mas-Muñoz *et al.*, 2013; Trong *et al.*, 2013; Freitas *et al.*, 2021). In line with this, estimates presented here fall on the upper end of this range (Table 4-4). These results indicate the FW-rearing environment influenced heritability estimates, where those of the loch-origin population were consistently greater than of RAS-reared counterparts. Importantly, the estimates for SW phenotypic traits contrast with the findings presented at the end of FW-rearing where, instead, the loch population demonstrated heritability estimates of approximately half of the RAS population (Table 4-3). This was suggested to be primarily a result of greater residual and phenotypic variance in the loch population (Chapter 2). Considering both results, since transfer to SW, heritability estimates appear to have increased in the loch but decreased in the RAS population.

Heritability is calculated from a ratio between additive genetic (V_g) and phenotypic (V_p) variance (Falconer, 1952; Falconer and Mackay, 1996; Gjedrem, 2000). As V_p is itself the sum of V_g and residual (V_r) variance (Falconer and Mackay, 1996), heritability estimates vary due to fluctuations in V_g , V_r , or both. Considering this, for the loch-reared population greater estimates of residual variance and coefficient of variance were observed for WBW and length, as was seen at the FW stage. However, it is of note that in both populations the coefficient of variance decreased from the FW to SW environment (Table 4-2 and Table 4-3). In contrast, estimates of the coefficient of genetic variance differed between FW and SW. Specifically, in FW, RAS-reared fish returned higher estimates than loch counterparts (Table 4-2), with the opposite true in SW (Table 4-3).

One factor that may have contributed to this observation is biased sampling. If present, this could have led to inaccurate representation of the true genetic variance present in the populations (Lage and Kornfield, 2006). In agreement, previous studies have demonstrated that removal of fish from tanks/pens can be biased due to a size-based distribution (Folkedal *et al.*, 2012; Nilsson *et al.*, 2013), size-based variability in being caught (Vindas *et al.*, 2016), and chance effects (Kritzer, Davies and Mapstone, 2001) (Nilsson and Folkedal, 2019). This bias could have been introduced at either FW or SW sampling and may have differed between husbandry environments (Nilsson and Folkedal, 2019). However, as only shared families were considered in the analysis, sampling bias may be less likely to have affected the results.

Alternatively, details of the experimental design could have additionally impacted these results. Specifically, at transfer to SW, all three tanks containing phenotypically female fish from the FW-RAS environment were pooled into a single sea-cage. This means that while estimates from the end of FW-rearing were derived from fish in only one of the three FW tanks, at three-months post-transfer, they were based on fish sampled from across all three. This contrasts with fish of loch-origin, where in both FW and SW environments, the population was only housed in a single tank/pen. This difference between populations may have helped drive the increase and decrease in loch and RAS populations post-transfer, respectively, in a similar manner to that described by Thorland *et al.* (2020).

4.5.4. Genotype-environment interactions (GxE)

In terms of genetic correlation (r_{gxe}), GxE is generally thought to be of significance to a program of selective breeding where this falls below 0.7 (Mulder *et al.*, 2006; Sae-Lim, 2013; Sae-Lim *et al.*, 2016). In line with this value, and as seen at the FW stage of development, SW GxE estimates were mostly insignificant, suggesting the best-performing families were the same in both SW populations (Table 4-5). Of note, r_{gxe} reported for phenotypic traits measured at SW (Table 4-5) were greater than at FW (Table 4-2). For example, though significant GxE was detected for WBW_{FW} , this was not seen in WBW_{SW} , suggesting the magnitude of GxE effects decreased once the population was transferred into a shared environment.

Considering these results, the strength of GxE effects can be attributed to (1) the degree of difference between environments (Mas-Muñoz *et al.*, 2013; Sae-Lim *et al.*, 2013; Turra *et al.*, 2016), and (2) the relative time spent in shared compared to separate rearing environments (Dupont-Nivet *et al.*, 2010; Agha *et al.*, 2018). Reflecting on this latter point, the RAS and loch populations were first co-reared in RAS for about nine-months, separately reared in their FW environments for about one-month and sampled after three-months in the same SW environment. Therefore, it is not unexpected that the estimates in SW GxE between phenotypic traits were low, given the limited time the populations were separately reared relative to the longer period they shared the same environment. In agreement, results have demonstrated the strength of GxE decreased with shorter periods of environment separation (Swan, Thompson and Ward, 2007). Similarly, Dupont-Nivet *et al.* (2010) reported greater genetic correlations (r_{gxe}) when populations had shared a longer period of communal rearing;

and conversely, Agha *et al.* (2018) reported greater estimates of GxE (lower r_{gxe}) in an experimental population that never shared an environment.

A notable exception to these generally high r_{gxe} was, however, SW GxE estimates for growth trait measurements (except for ADG_{WBW}) (Table 4-5), which fell below the 0.7 benchmark (Sae-Lim, 2013). These results infer that contrary to phenotypic traits, family growth performance in SW was impacted by the FW-rearing environment. The strength of GxE estimates can additionally be influenced by the trait of interest. Specifically, while phenotypic traits are affected by the accumulated environmental experience since fertilization/conception, growth traits can be calculated across specific periods and in turn reflect the environmental effects of this time. As a result, previous studies have reported significant GxE for growth traits that are not seen in the overall phenotypic performance (Dupont-Nivet *et al.*, 2008, 2010; Omasaki *et al.*, 2016). While these have typically calculated growth over the period spent in separate rearing environments, here growth was instead measured after the environment-specific populations have been returned to a shared location. Despite this difference, the results presented similarly show the presence of GxE for growth, but not for phenotypic measurements.

Estimated genetic correlations for FW-SW GxE were generally high in both RAS and loch populations, suggesting the best performing families were the same pre- and post- transfer (Table 4-6). However, this was at the exception of the correlation between WBW measured in FW- and SW-Loch populations, which at 0.57 (s.e 0.24) fell below the 0.7 threshold (Sae-Lim, 2013). While this agrees with previous studies which have identified genetic variance in growth performance throughout the production cycle (Gjerde, Simianer and Refstie, 1994; Kause *et al.*, 2003; Thorland *et al.*, 2020) it contrasts with what was expected. Specifically, as the loch environment is, in theory, more similar to that of the SW site in comparison to RAS, weaker GxE may have been expected. In agreement with this, mean and standard deviation of the loch environment was more like that of the SW site (Figure 2-1 and Figure 4-2). It should be noted that a comparison could only be made for temperature. Therefore, other variables, which cannot be considered here, could have differed between environments and contributed to the greater re-structuring of family weight performance in the loch population post-transfer.

It is worth noting that r_{gxe} estimates could have been biased due to sampling size. Specifically, based on suggestions in Sae-Lim, Komen and Kause (2010), while number of families was sufficient (124), both the number of fish sampled per environment (650-910) and number of fish per family (~6) were below recommendations. Consequently, the quantification of GxE effects presented here could have been downwardly biased. In evaluation of GxE for growth, it should also be considered that as derived/composite traits, they may be more likely to express GxE, as has been suggested previously (Evans and Langdon, 2006).

4.5.5. Aquacultural implications

Results demonstrated that at the end of FW-rearing, RAS-reared smolts were smaller than their loch counterparts. This may have been a result of environmental differences between them, in particular the high density of RAS-rearing. However, despite their smaller size, the RAS-origin smolts showed enhanced growth rate post-SW transfer. Given the suggestion that early SW growth trajectories continue to harvest (Lai *et al.*, 2024) it is possible that the difference in size between the RAS and loch populations would diminish with time. This, combined with low mortality rate reported in both populations post-transfer suggests the size difference at the end of FW-rearing may have limited production implications.

Additionally, at both time points the RAS population demonstrated lower trait heterogeneity, with indications of sexually dimorphic growth also present in the loch. Notably, this homogeneous RAS growth was maintained even after the population was transferred to the SW environment. This finding is particularly advantageous commercially as trait homogeneity can improve animal welfare, increase produce value, and reduce production costs (Janhunen *et al.*, 2012; Marjanovic *et al.*, 2016; Vu *et al.*, 2021). While this could be both genetically and environmentally regulated, it is suggested here that the same RAS specific variables that contributed to their small size may have also encouraged trait homogeneity. Management of FW-rearing environment should then consider the balance between FW size and trait variance.

Estimated parameters and GxE quantification indicated different patterns of genetic variance between time points dependent on FW-rearing. Firstly, heritability estimates for the loch population increased from pre- to post-transfer, but a low genetic correlation between FW and

SW WBW was identified. The opposite was true of the RAS population. These results could be used to help improve breeding strategies. For example, the difference in heritability estimates suggest that selection should target different time points depending on the FW-rearing environment employed. In line with results presented here, this would be SW traits for the loch-reared population, and those at the end of FW-rearing for the RAS fish. Of note, because of the higher genetic correlation between FW and SW traits in the RAS populations, selection applied at FW would be expected to also produce a large response in SW traits.

Lastly, growth performance appears to be environment-specific with moderate-to-strong GxE identified between SW-RAS and SW-Loch populations. This suggests that targeting environment-specific growth in a selection program could improve post-SW transfer performance. Given indications of enhanced growth rates in the RAS population post-transfer, selection for growth could be particularly effective in this population.

4.6. Conclusions

Though smaller at the end of FW-rearing, RAS-reared post-smolts were growing at a faster rate in SW than loch-origin counterparts. RAS-origin post-smolts also demonstrated lower trait variance than loch counterparts, with the latter also showing indication of sexual dimorphic growth. In the loch population, heritability increased from pre- to post-SW transfer. The opposite was true in RAS. In addition, while weak-to-moderate GxE was detected overall, significant re-ranking was identified for SW growth traits.

Chapter 5-General Discussion

5.1. Introduction

Employment of recirculating aquaculture systems (RAS) is increasing (Bergheim *et al.*, 2009). While RAS offer advantages (Ebeling and Timmons, 2012; Murray, Bostock and Fletcher, 2014), they provide novel environmental characteristics to housed populations which differ from other culture systems (Bergheim *et al.*, 2009; Schumann and Brinker, 2020; Lai *et al.*, 2024). Considering this, anecdotal evidence from the industry has suggested poor post-saltwater (SW) transfer performance of RAS-reared Atlantic salmon. Equally, empirical studies have identified molecular and physiological differences in RAS-reared salmon at the end of freshwater (FW) development, relative to other culture systems (Roque d'Orbcastel *et al.*, 2009; Kolarevic *et al.*, 2014; van Rijn *et al.*, 2020; Lai *et al.*, 2024).

In addition to the environmental variables, salmon performance under RAS conditions may be affected by genetic factors. For example, in Scotland, the predominant FW culture system is still net-pens in lochs (Clarke and Bostock, 2017; Regan *et al.*, 2021), and performance of stocks in such an environment may have been factored into selective breeding programs. However, genotypic performance of current commercial populations in novel RAS environments, relative to more conventional culture systems, has rarely been investigated (e.g. Sae-Lim *et al.*, 2013; Gonzalez, Gallardo-Hidalgo and Yáñez, 2022). Understanding of genotypic sensitivity to rearing environments was therefore needed to help guide wider implementation of RAS.

Given its sensitivity to the environment, alterations to the epigenome adds another level of complexity to this picture (Bollati and Baccarelli, 2010; Taudt, Colomé-Tatché and Johannes, 2016). For example, DNA methylation (DNAm) changes have been reported in many salmonids in response to hatchery rearing (Le Luyer *et al.*, 2017; Rodriguez Barreto *et al.*, 2019; Wellband *et al.*, 2021), or specific environmental variables (Marandel *et al.*, 2016; Burgerhout *et al.*, 2017; Uren Webster *et al.*, 2018). Due to the regulatory effect of DNAm on gene expression (Ambrosi, Manzo and Baubec, 2017), the use of RAS may drive alterations to the epigenome with potential implication for Atlantic salmon FW development and SW performance.

Considering the role the environment plays in Atlantic salmon FW development (Stefansson *et al.*, 2008), this thesis hypothesised that the RAS environment may exert an impact on parr with regards to their preparedness for SW transfer and later life successes. To facilitate the growing use of RAS, a better understanding of the environmental, genetic and epigenetic factors on salmon development under varied culture systems was urgently needed. The overarching aim of this work was, therefore, to contribute to bridging this knowledge gap by investigating genotype-environment interactions (GxE) between a RAS- and loch-reared commercial population as well as DNAm changes in response to the RAS environment. Specific research objectives were as follows,

(1) to investigate the comparative performance of current commercial stocks in RAS to help inform future breeding decisions (**Chapter 2**),

(2) to study the impacts of environmental variables differing between FW-rearing systems on DNA methylation and gene expression patterns (**Chapter 3**), and

(3) to assess the impact of RAS-rearing on Atlantic salmon SW performance, through assessment of genotype-environment interactions (**Chapter 4**).

The results gathered have provided insight and guidance into husbandry and breeding decisions in the context of wider use of RAS for Atlantic salmon production.

5.2. Key results

In **Chapter 2** the performance of current commercial stocks reared in RAS was compared to loch populations. To achieve this, and while working within a commercial setting, a population of Atlantic salmon were co-reared for ~ nine-months, at which point they were evenly split between net-pens in an ambient loch environment or RAS. After ~eight weeks of separate rearing leading up to SW transfer and smoltification, both populations were sampled. To answer the wider aim of Chapter 2, specific objectives were targeted with key results as follows.

Phenotypic and genetic parameters within each rearing environment were estimated with results indicating that although smaller in terms of weight and length, fish reared in the RAS

environment demonstrated greater condition factor (Table 5-1). Yet, despite their smaller size, RAS-reared smolts demonstrated consistently lower trait variance (Table 5-1). In part a result of this lower trait variance, heritability estimates were greater in RAS, where these were nearly twice that of the loch population (Table 5-1). Secondly, genotype-environment interactions (GxE) between RAS- and loch-reared populations were quantified as r_{gxe} - the genetic correlation between a trait measured in each environment. While r_{gxe} estimates for length and condition factor suggested GxE effects were weak, for WBW these fell below previously identified thresholds (Table 5-2). Lastly, genetic markers associated with trait expression between environments were investigated from genome wide association study (GWAS). Results indicated that while a general pattern of polygenic regulation was identified, stronger genetic associations in the RAS compared to loch environment as well as suggestions of environment-specific regions in significant association with trait expression were also identified.

Table 5-1 Mean (standard deviation, s.d) and narrow sense heritability (h^2) (standard error, s.e) for freshwater (FW) and saltwater (SW) traits, information combined from Table 2-2, Table 4-3 and Table 4-4. WBW is whole-body weight, K is condition factor, ADG is average daily gain, and SGR is specific growth rate.

	Mean (s.d)		h^2 (s.e)	
	RAS	Loch	RAS	Loch
WBW_{FW} (g)	88.03 (17.87)	121.87 (27.11)	0.42 (0.55e ⁻¹)	0.18 (0.43e ⁻¹)
Length_{FW} (cm)	19.05 (1.25)	21.35 (1.67)	0.40 (0.55e ⁻¹)	0.15 (0.43e ⁻¹)
K_{FW}	1.26 (0.08)	1.24 (0.11)	0.37 (0.52e ⁻¹)	0.13 (0.42e ⁻¹)
WBW_{SW} (g)	738.9 (119.82)	826.25 (169.19)	0.24 (0.55e ⁻¹)	0.34 (0.56e ⁻¹)
Length_{SW} (cm)	37.33 (1.65)	38.63 (2.19)	0.20 (0.50e ⁻¹)	0.35 (0.56e ⁻¹)
K_{SW}	1.41 (0.14)	1.41 (0.13)	0.16 (0.48e ⁻¹)	0.24 (0.51e ⁻¹)
ADG_{WBW} (g/day)	6.17 (1.11)	6.19 (1.45)	0.23 (0.54e ⁻¹)	0.35 (0.56e ⁻¹)
ADG_{LENGTH} (cm/day)	0.18 (1.58e ⁻²)	0.15 (1.92e ⁻²)	0.24 (0.50e ⁻¹)	0.42 (0.55e ⁻¹)
SGR_{WBW} (%g/day)	2.05 (0.18)	1.70 (0.20)	0.46 (0.57e ⁻¹)	0.51 (0.52e ⁻¹)
SGR_{LENGTH} (%cm/day)	0.65 (5.05e ⁻²)	0.53 (5.95e ⁻²)	0.47 (0.55e ⁻¹)	0.59(0.49e ⁻¹)

Chapter 3 aimed to investigate how the impact of environmental variables, typical of RAS, impact on the epigenome. This was achieved by exposing a population of Atlantic salmon in their FW phase to one of six treatments, a combination of photoperiod (continuous light or light and dark cycles), feeding regime (continuous feeding or feeding in the perceived day), and temperature (high or low temperature), from which reduced representation bi-sulphite

sequencing (RRBS) and RNA-seq was performed on brain samples. From this, specific objectives were investigated and key results observed.

Initially, patterns in the brain methylome were characterised, revealing a bi-modal distribution of CpG methylation. While this was consistent between genomic features and largely similar between treatment groups, CpG sites falling within the 1st exon or promoter-TSS showed a tendency towards hypo-methylation. Secondly, differentially methylated cytosines (DMC) and differentially expressed genes (DEG) in response environmental manipulation were identified and characterised. It was observed that on the level of individual CpG sites there was little indication of treatment related differences, though this result could have been affected by methodological and analytical approaches taken. In contrast, transcriptomic results demonstrated differences, both at global and site-specific scales, in relation to photoperiod and feeding regime manipulation. While in both temperature groups, photoperiod effects on gene transcription were clearly seen, this was more apparent at high temperature. Furthermore, enrichment analysis of DEG indicated treatment related differences could potentially be associated with altered patterns of metabolic activity and neuronal development, amongst others. Thirdly, the link between DNAm and gene expression was investigated and while overarching correlations were identified between gene expression and DNAm, treatment related differences in DNAm could not be widely related to gene expression.

Lastly, **Chapter 4**, which followed on from Chapter 2, aimed to characterise the influence of FW-rearing environments on salmon's longer-term performance to verify if hypothesised changes to genetic or epigenetic performance in FW had an impact in SW. To do this, after FW sampling (explored in Chapter 2), both FW-Loch and -RAS populations were transferred into the same SW cage site for grow-out, where fish of both FW-origins were sampled three-months after. From this, the specific objectives were investigated with key results as follows. Initially, the impact of FW-origin on SW performance was evaluated. Results indicated that although smaller on SW transfer, fish of RAS-origin were increasing in weight and length at a comparatively faster rate. Furthermore, RAS-origin fish still demonstrated lower trait variance. Secondly, the genetic architecture and GxE interactions underlying SW traits were investigated. Results indicated SW heritability estimates were greater in the loch compared to

RAS environment, suggesting that in the former this increased on SW transfer, with the opposite true of the RAS population (Table 5-1). Furthermore, while moderate-to-weak GxE were identified for SW phenotypic traits, moderate-to-strong estimates of GxE were observed for SW growth traits. Lastly, how well FW performance predicts SW outcomes was assessed through GxE quantification, with weak GxE identified at the exception of weight measured at FW and SW in the loch population (Table 5-2).

Table 5-2 Summary of all quantification of GxE (r_{gxe}) where y1 is trait 1, and y2 trait 2. Information combines that presented in Chapter 2 and 4, as well as Table 4-5 and Table 4-6. WBW is whole-body weight, K is condition factor, ADG is average daily gain, SGR is specific growth rate, and s.e is standard error. Those falling below 0.7 benchmark, as defined in Sae-Lim *et al.* (2016), indicated by *.

y1	y2	r_{gxe} (s.e)
WBW _{FW, RAS}	WBW _{FW, LOCH}	0.62 (0.14)*
Length _{FW, RAS}	Length _{FW, LOCH}	0.78 (0.15)
K _{FW, RAS}	K _{FW, LOCH}	0.85 (0.14)
WBW _{SW, RAS}	WBW _{SW, LOCH}	0.86 (0.10)
Length _{SW, RAS}	Length _{SW, LOCH}	0.97 (9.00e ⁻²)
K _{SW, RAS}	K _{SW, LOCH}	0.93 (0.15)
ADG _{WBW, RAS}	ADG _{WBW, LOCH}	0.87 (9.90e ⁻²)
ADG _{LENGTH, RAS}	ADG _{LENGTH, LOCH}	0.60 (0.13)*
SGR _{WBW, RAS}	SGR _{WBW, LOCH}	0.21 (0.11)*
SGR _{LENGTH, RAS}	SGR _{LENGTH, LOCH}	0.17 (0.10)*
WBW _{FW, RAS}	WBW _{SW, RAS}	0.82 (0.11)
WBW _{FW, LOCH}	WBW _{SW, LOCH}	0.57 (0.24)*
Length _{FW, RAS}	Length _{SW, RAS}	0.82 (0.13)
Length _{FW, LOCH}	Length _{SW, LOCH}	0.78 (0.34)
K _{FW, RAS}	K _{SW, RAS}	0.78 (0.15)
K _{FW, LOCH}	K _{SW, LOCH}	0.85 (0.49)

5.3. Study conclusions and aquacultural implications

This thesis has provided key information that extends our understanding of how the use of novel husbandry environments can impact commercial aquaculture production. Specifically, while previous studies have compared the performance of species under RAS conditions to other culture systems (e.g. Saillant *et al.*, 2006; Mas-Muñoz *et al.*, 2013; Fernandes *et al.*, 2019), similar studies have only rarely been performed in salmon (e.g. Correa *et al.*, 2018; Gonzalez, Gallardo-Hidalgo and Yáñez, 2022). Furthermore, while the latter researched the comparative performance of salmon reared under RAS conditions throughout the production cycle, how its use as an early husbandry environment could impact on later life success had not been

thoroughly explored. Similarly, while studies have compared DNAm patterns of salmonids reared in hatchery environment to those of wild or semi-natural origin (e.g. Le Luyer *et al.*, 2017; Gavery *et al.*, 2018), analogous research in an aquaculture context had rarely been performed. The work presented here has addressed these gaps while conducting research in a complex commercial setting and taking a powerful approach that combines phenotypic, genotypic, epigenomic and transcriptomic data.

Based on previous studies which observed negative consequence so RAS-rearing on salmon performance (Ytrestøyl *et al.*, 2022; Pino Martinez *et al.*, 2023; Lai *et al.*, 2024), as well as differential methylation due to early rearing environments (e.g. Le Luyer *et al.*, 2017; Gavery *et al.*, 2019; Venney, or Wellband and Heath, 2020), it was hypothesised that maladaptive epigenetic programming or priming shaped by the RAS environment may be the mechanism underpinning anecdotal suggestions that the SW performance of RAS-reared fish is below that of loch-origin fish (Gavery and Roberts, 2017; Le Luyer *et al.*, 2017; Roy *et al.*, 2021). However, results of this thesis did not support this theory. Specifically, these findings suggested that although phenotypic performance differed at the end of FW phase depending on the rearing environment, this did not negatively impact the RAS-reared fish post-SW transfer. Additionally, increased homogeneity of the RAS-reared population in both the FW and SW environment was observed which, importantly, has commercial and practical advantages in terms of market value, production cost and animal welfare (Janhunen *et al.*, 2012; García-Ballesteros *et al.*, 2021; Garcia *et al.*, 2021). Notably, despite these phenotypic differences at FW, as well as gene expression differences in response to environmental manipulation, meaningful changes to DNAm were not observed, suggesting a possible role of alternative epigenetic mechanisms, as has been observed previously (e.g. Harvey *et al.*, 2024).

Overall, the above findings suggests that use of RAS for the FW stage of commercial populations rearing is not of significant concern to their performance and even carries advantages over that of loch systems (i.e. homogeneity). However, differences observed in genetic parameters depending on the FW-rearing environment, as well as variable quantification of GxE effect (r_{gxe}), suggests these could be considered in breeding and management decisions to improve productivity and efficiency. For example, contrasting

patterns of trait heritability suggest selection for phenotypic traits measured at the end of FW-rearing would be most efficient in the RAS population, whereas this would be at SW for the loch population. However, consistently high genetic correlations (r_{gxe}) between FW and SW traits observed in the RAS-reared population (but not the loch-reared) infers selection at either time point would drive a strong correlated response at the other, benefiting growth throughout the production cycle.

Additionally, between RAS-reared and loch-reared populations, GxE estimates below current benchmarks (Sae-Lim, 2013; Sae-Lim *et al.*, 2016) were observed for weight at the end of FW-rearing, as well as SW growth. To avoid the loss of genetic gains this can cause, different approaches could be taken to account for significant GxE, such as husbandry and management decisions to minimise environmental differences between farm sites (Sae-Lim, 2013). In the current study, greater estimates of residual variance at both the FW and SW time points were observed in the loch-reared population. To address significant re-ranking in FW body weight, this result suggested environmental manipulation should target the FW-loch environment. Though, the limited husbandry control inherent to net-pens floating in lochs may prohibit the practicality of this. Conversely, while the RAS environment could be more easily altered to resemble that of the FW-loch, this would sacrifice the benefits (as described above) it offers.

Alternatively, GxE can be accounted for by re-locating breeding programs to the environment which yields the greatest overall or average genetic gains across farming locations (Sae-Lim, 2013). Other approaches include the use of index selection, which considers phenotypic performance at the nucleus/breeding population and sib-performance at farms, or the establishment of environment-specific selection programs (Mulder and Bijma, 2005; Sae-Lim, 2013). While these latter options offer greater potential for genetic gain across farming sites, they incur significant economic investment. Investigation into the cost-benefit trade-off, modulated further by the level of market competition, would be needed before any decisions are made (Mulder and Bijma, 2005; Sae-Lim, 2013).

5.4.Limitations and future directions

5.4.1.Studying GxE in a commercial setting

Despite these findings, the limitations in analytical and methodological decisions must be considered in the evaluation of results. The trial which formed the basis for Chapters 2 and 4 took place under industry conditions as well as working on a commercial population. As phenotypic and genetic performance are both population- and environment-specific (Powell *et al.*, 2008; Garber *et al.*, 2019), this approach meant insights gained will have direct relevance to, and practical implications for, farm management (Jerry *et al.*, 2012). As a consequence, however, many aspects of the experimental design were not under experimental control, as well as some information not being available for analysis. This placed limitations on the research carried out.

Firstly, the population was not tracked prior to their split between FW environments. This means it was unknown if systematic differences existed between families, or if there was the period of separate family rearing. If present, then genetic parameters observed could have been biased (Castillo-Juárez *et al.*, 2007; Dupont-Nivet *et al.*, 2010). Some caution should therefore be paid to the results. Future studies should also focus on trying to ensure populations are tracked throughout production. Similarly, because of biomass limitations at the sea-cage site, to ensure welfare of the populations, environment-specific populations had to be pooled prior to harvest. While this was unavoidable, as data was not available through to harvest, many inferences made on the impact of FW-rearing environment for salmon husbandry and breeding decisions included a level of speculation. It would be useful in the future to study populations through the full production cycle. Furthermore, for similar reasons, the phenotypically male populations for each FW site had to be pooled upon SW transfer. This reduced the population size used in Chapter 4 analysis, potentially biasing results (Sae-Lim, Komen and Kause, 2010).

Working within a commercial setting also had implications for the availability of environmental data. Specifically, detail and range of environmental parameters monitored across environments were limited, particularly related to the FW-loch, and number of parameters shared between FW and SW time points. In response, the approach chosen

modelled the holistic environment, meaning that the specific environmental variables potentially driving observed genetic and phenotypic differences, as well as GxE, could not be analytically studied (Sae-Lim *et al.*, 2014, 2016; Sae-Lim, Mulder, *et al.*, 2015). Considering this, between FW-rearing environments there was a marked difference in stocking density. As this has been previously implicated in growth performance (Liu *et al.*, 2014; Liu, Liu and Sun, 2017; Wang *et al.*, 2019), this was suggested as a possible factor underpinning observed differences in phenotypic performance, genotypic parameters as well as GxE between RAS and loch. While previous studies have compared holistic environments which differ in a range of parameters including density (e.g. Sae-Lim, Kause, *et al.*, 2015), research directly investigating its effect has only been performed widely in shrimp (*Penaeus japonicus* and *Penaeus vannamei*) (e.g. Ibarra and Famula, 2008; Nguyen, Ninh and Hung, 2020; García-Ballesteros *et al.*, 2021). Given the results presented in Chapter 2, such research in salmonids may therefore be worth conducting.

If available, such data could have also provided greater insight into environmental characteristics, potentially providing clarity into why there was significant tank effect nested within environment in the loch but not RAS environment, for example. Lastly, while increased trait homogeneity has been linked to environmental stability in RAS previously (e.g. Vu *et al.*, 2021), with similar suggestions stated here, additional environmental data could have aided in verification of this theory. Further study into this may also be warranted. Considering this, measurements of residual variance indicated differences in management of trait heterogeneity between RAS and loch environments. However, future analyses focusing specifically on the genetic underpinnings of residual variance between FW environments could provide stronger evidence for how to control this (Janhunen *et al.*, 2012; Sonesson, Ødegård and Rönnegård, 2013; Iung *et al.*, 2020).

It is also worth mentioning that there is no standard 'RAS environment'. Instead, differences will exist in terms of size, husbandry decisions, and technical specifications, for example (Ebeling and Timmons, 2012). Therefore, while results presented here do show similarity with previous research on the comparative performance of RAS-reared populations (Sae-Lim *et al.*, 2014; Van Sang *et al.*, 2020), contradicting findings have also been found (Dupont-Nivet *et al.*, 2008; Dupont-Nivet *et al.*, 2010; Mas-Muñoz *et al.*, 2013; Li *et al.*, 2019). In the future, a

comprehensive review of the RAS environment and its variability may aid in understanding how widely applicable current observations of RAS-rearing's influence on phenotypic and genetic parameters are. Similarly, the data provided here was only for a single generation, single year group, and at a single farm site. Previous research has shown that genetic parameters of the same genetic stock reared in the same environment varied between years (e.g. Gonzalez, Gallardo-Hidalgo and Yáñez, 2022). Therefore, it would be recommended to repeat such analysis to try and identify how consistent, or not, the impact of alternative FW-rearing environments is.

5.4.2. Sampling bias and family representation

Beyond the limitations of working in a commercial setting, the sample size and approach to fish selection in the GxE study could have influenced observations. Firstly, the number of fish sampled here was limited via the trade-off between genotyping costs and the desire to accurately capture population variability. As a result, the total number of fish sampled, as well as the number per family, were below previously suggested thresholds (Sae-Lim, Komen and Kause, 2010), potentially downwardly biasing r_{gxe} . By contrast, the number of families sampled were above minimum number recommended (Sae-Lim, Komen and Kause, 2010; Sae-Lim *et al.*, 2016). Future studies could, therefore, target sampling to reduce the number of families sampled but increase their size. This could boost robustness of estimates, especially in the face of economic limitations of genotyping. Other approaches to increase the sample size could be to genotype fish to a lower density and employ imputation technologies to then enable detailed quantitative genetic analysis (Yoshida *et al.*, 2018).

Additionally, sampling was performed via random netting of individuals from tanks/pens/cages. It should be noted that salmon have been seen to demonstrate size based spatial distribution, which may also differ between types of husbandry environment. Therefore, between FW-environments, as well as with the sea-cage site, sampling may have captured biased representations of the population (Nilsson *et al.*, 2013; Nilsson and Folkedal, 2019). Anecdotal evidence of this in our sampling practice is that during sampling of the sea-cage site, on occasion the same fish was randomly captured more than once (though only sampled once). Investigation and consideration of the best practices for random sampling should be more carefully considered in the design of a future study.

5.4.3. Epigenetic study approaches

Firstly, while the intention of Chapter 3 was to tease apart the effect of different variables often manipulated in a RAS environment, during analysis it became apparent that this, along with other analytical approaches taken, may have been overcomplicated and limited the results and insight gathered. Specifically this includes (1) the time in which the environmental manipulation was applied (Anastasiadi, Díaz and Piferrer, 2017; Kho *et al.*, 2024), (2) the choice of complex brain tissue (Gavery *et al.*, 2018; Wellband *et al.*, 2021), (3) nature of the environmental treatment applied (Migaud, Cowan, *et al.*, 2007; Vera *et al.*, 2010), (4) the analytical approach chosen (Kurdyukov and Bullock, 2016; Geistlinger *et al.*, 2021; Wellband *et al.*, 2021), as well as (5) a lack of genotypic/family information (Taudt, Colomé-Tatché and Johannes, 2016; Hu *et al.*, 2021). Furthermore, an oversight in the study design was not to take assessments of sex, maturation, or smoltification. In particular, the latter two both occur during salmon's FW stage (Klemetsen *et al.*, 2003; Stefansson *et al.*, 2008), are effected by the environmental variables manipulated in this study (Thrush, Duncan and Bromage, 1994; Björnsson *et al.*, 2000; Berrill *et al.*, 2003; Fjellidal, Hansen and Huang, 2011), and previously demonstrated to have both epigenetic and gene expression impacts (Morán and Pérez-Figueroa, 2011; Robertson and McCormick, 2012; Mobley *et al.*, 2021). If assessed during the trial, greater confidence could have been made into their (possible) confounding effect on the results presented here.

Secondly, during planning it was chosen to measure time in degree days due to rearing the population at two different temperatures. The intention was to minimise developmental and physiological differences between groups in response to the effect of temperature on the rate of such processes, as previously seen (McCormick, Moriyama and Björnsson, 2000; McCormick *et al.*, 2002; Imsland, Handeland and Stefansson, 2014). It became clear, however, that over the exposure period, due to the difference in actual days between sampling in the high and low temperature group, a large difference in size, and possibly also development, had evolved. The approach to independently analyses temperature group samples was therefore taken. This decision created a novel problem for analysis where no 'true' baseline, matching control conditions at the end of the experimental period, existed for the high temperature group. This may have influenced the pattern of gene expression and DNAme

seen through time, and at the end of the experimental period between treatment groups. Importantly, between temperature groups, contrasting patterns of gene expression were observed. Namely, at low temperature a significant number of differently expressed genes (DEG) were observed through time, but few were seen between treatments, with the opposite seen at high temperature. It would, therefore, be interesting to further investigate this pattern to ascertain whether it is a result of the analytical approach, or if the use of alternative rearing temperatures during this developmental period has the biological impact on gene expression observed here.

5.5.Thesis conclusions

Across three research chapters, this work has addressed the overarching thesis aim, providing insight into the environmental, genetic and epigenetic impacts of FW-rearing in RAS on Atlantic salmon performance. The first research objective was achieved by providing evidence of the phenotypic and genetic factors of the RAS environment at the FW stage. Specifically, although RAS-reared Atlantic salmon were smaller in size, they demonstrated lower trait variance and greater heritability estimates. These differences were suggested to be a result of differing variables between the two environments, and in particular the effect of stocking density and water recirculation, which deserve further attention. Evidence was also provided that genotypic performance was environmentally sensitive, with indications that traits were underpinned by different genomic associations depending on rearing environment. Further investigation of genetic regulation of environment-specific traits maybe warranted.

The second research objective was achieved by identifying DNAME and gene expression changes in response to environmental manipulation often applied in RAS. Contrary to my expectation, outputs demonstrated that manipulation of photoperiod, temperature or feeding regime did not impact the epigenome in a meaningful way. This finding did not support the theory that use of RAS system is inappropriately shaping epigenetic patterns regarding FW development and preparedness for SW transfer. Despite this, gene expression changes were detected, suggesting a possible role of alternative epigenomic mechanisms. Though this study was limited by methodological choices, the findings suggested future research directions, particularly into unexpected patterns in gene expression.

The last research objective was met by investigating the longer-term impacts of FW-rearing in RAS on salmon's genetic and phenotypic performance. Unexpectedly, despite their smaller size on transfer, RAS-reared fish did not demonstrate poorer performance post-transfer. Instead, these fish were found to be increasing in size at a faster rate than loch-reared counterparts, all while maintaining greater trait homogeneity as seen in FW. Results indicated differences in genetic architecture of traits between RAS- and loch-origin populations. Patterns observed between FW and SW time points also differed between populations depending on their FW-origin. These findings suggested FW-rearing in RAS did not negatively impact long-term growth on salmon post-smolts. However, differences in genetic parameters between environment-specific population could be targeted to increase productivity throughout the production cycle.

Appendix A.

A. 1 The number of genotypic (based on SDY gene expression) and phenotypic (based on ultrasound detection of testis) males and females at the FW sampling, split by FW environment.

	M _{Genotype}	M _{Phenotype}	F _{Genotype}	F _{Phenotype}
RAS	732	245	313	664
Loch	393	245	572	720
Total	1057	490	885	1452

A. 2 Mean, standard deviation (s.d) and range for additional water quality parameters measured in the freshwater RAS environment over the period of separate rearing. TAN is total available ammonia.

<i>Parameter</i>	Mean (s.d)	Range
Turbidity (NTU)	1.07 (0.85)	(0.08-3.88)
TAN (mg/L)	0.070 (0.071)	(0.00-0.28)
Nitrite (mg/L)	0.033 (0.052)	(0.00-0.23)
Nitrate (mg/L)	235.61 (63.61)	(90.00-357.00)
Toxic ammonia (NH₃) (mg/L)	0.00022 (0.00027)	(0.00-0.0012)
CO₂ (mg/L)	5.57 (1.80)	(3.75-12.75)
Total Alkalinity (mg/L)	108.30 (45.36)	(30.00-210.00)
Calcium hardness (mg CaCO₃/L)	30.30 (18.05)	(0.00-58.00)
Total hardness (mg CaCO₃/L)	61.80 (25.97)	(5.00-115.00)

A. 3 Number of SNPs removed in quality control for the parental and offspring genotypes.

	Step	Parents	Offspring
	Start	55357	65774
SNPs removed	<i>Dup/chr30</i>	506	785
	<i><10% SNP</i>	106	1146
	<i><10% individ</i>	0	0
	<i>HWE</i>	54	3143
	<i>MAS</i>	1202	1122
	End	53489	59578

A. 4 Genomic distribution of filtered SNPs common to both parental and offspring arrays that were retained for analysis.

Chr.	SNP number	Coverage (kb)
1	3,401	158,976
2	1,210	72,910
3	2,139	92,338
4	1,846	82,398
5	1,847	80,317

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6	1,842	87,014
7	1,317	57,626
8	396	26233
9	2,532	141,543
10	2,508	116,086
11	1,773	93,888
12	1,815	91,863
13	2,281	107,731
14	2,038	93,886
15	1,949	103,896
16	1,554	87,455
17	1,091	57,656
18	1,193	70,695
19	1,467	82,833
20	1,846	86,732
21	1,153	57,906
22	1,355	63,060
23	1,303	49,692
24	1,143	48,558
25	1,037	51,339
26	900	47,201
27	1,122	43,591
28	922	39,329
29	781	42,455

A. 5 Parameter estimates from genomic analysis of trait (whole-body weight (WBW, g), length (cm), and condition factor (K)) architecture performed using pedigree (A matrix) information only. V_r is the residual variance, V_g the genetic variance, h^2 the narrow sense heritability and s.e standard error.

	WBW (g)		Length (cm)		K	
	RAS	Loch	RAS	Loch	RAS	Loch
V_r (s.e)	173.70 (18.96)	569.89 (42.10)	0.85 (0.85)	2.35 (0.16)	0.35e ⁻² (0.38e ⁻³)	0.10e ⁻¹ (0.67e ⁻³)
V_g (s.e)	129.35 (26.64)	164.11 (45.35)	0.52 (0.11)	0.44 (0.15)	0.28e ⁻² (0.54e ⁻³)	0.16e ⁻² (0.61e ⁻³)
h^2 (s.e)	0.42 (0.75e ⁻¹)	0.22 (0.58e ⁻¹)	0.38 (0.72e ⁻¹)	0.16 (0.52e ⁻¹)	0.44 (0.73e ⁻¹)	0.13 (0.51e ⁻¹)

A. 6 Parameter estimates from genomic analysis of trait (whole-body weight (WBW, g), length (cm), and condition factor (K)) architecture performed using genomic relationship matrix (GRM) information only. V_r is the residual variance, V_g the genetic variance, h^2 the narrow sense heritability and s.e standard error.

	WBW (g)		Length (cm)		K	
	RAS	Loch	RAS	Loch	RAS	Loch
V_r (s.e)	171.41 (13.65)	588.48 (35.65)	0.81 (0.63e ⁻¹)	2.32 (0.14)	0.37e ⁻² (0.27e ⁻³)	0.10e ⁻¹ (0.60e ⁻³)
V_g (s.e)	131.34 (21.86)	133.63 (34.43)	0.57 (0.99e ⁻¹)	0.44 (0.13)	0.23e ⁻² (0.40e ⁻³)	0.15e ⁻² (0.52e ⁻³)
h^2 (s.e)	0.43 (0.56e ⁻¹)	0.18 (0.45e ⁻¹)	0.41 (0.57e ⁻¹)	0.16 (0.44e ⁻¹)	0.38 (0.54e ⁻¹)	0.14 (0.43e ⁻¹)

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A. 7 Genetic (r_g) and residual (r_r) correlations within environments for analysis of traits (whole-body weight (WBW, g), length (cm), and condition factor (K)) performed using pedigree (A matrix) information only. Results significantly different from zero in bold. s.e is standard error.

	Environment	r_g (s.e)	r_r (s.e)
<i>WBW-Length</i>	RAS	0.94 (0.20e ⁻¹)	0.95 (0.79e ⁻²)
	Loch	0.96 (0.10)	0.92 (0.77e ⁻¹)
<i>WBW-K</i>	RAS	0.41 (0.13)	0.24 (0.73e ⁻¹)
	Loch	0.58 (0.29)	-0.43e ⁻¹ (0.50e ⁻¹)
<i>Length-K</i>	RAS	0.69e ⁻¹ (0.15)	-0.50e ⁻¹ (0.75e ⁻¹)
	Loch	0.35 (0.40)	-0.42 (0.42e ⁻¹)

A. 8 Genetic (r_g) and residual (r_r) correlations within environments for analysis of traits (whole-body weight (WBW, g), length (cm), and condition factor (K)) performed genomic relationship matrix (GRM) information only. Results significantly different from zero in bold. s.e is standard error.

	Environment	r_g (s.e)	r_r (s.e)
<i>WBW-Length</i>	RAS	0.95 (0.13e ⁻¹)	0.94 (0.62e ⁻²)
	Loch	0.94 (0.37e ⁻¹)	0.92 (0.68e ⁻¹)
<i>WBW-K</i>	RAS	0.35 (0.11)	0.28 (0.50e ⁻¹)
	Loch	0.28 (0.24)	0.14e ⁻¹ (0.43e ⁻¹)
<i>Length-K</i>	RAS	0.39e ⁻¹ (0.13)	-0.36e ⁻¹ (0.54e ⁻¹)
	Loch	-0.44e ⁻¹ (0.26)	-0.37 (0.37e ⁻¹)

A. 9 Lambda values (median(χ^2) / expected median(χ^2)) for whole-body weight (WBW, g), length (cm) and condition factor (K), for RAS and loch populations.

	WBW	Length	K
<i>RAS</i>	1.38	1.34	1.11
<i>Loch</i>	1.13	1.11	1.08

A. 10 From GWAS, additional information of SNPs found in significant association with whole-body weight (WBW), length, and condition factor (K) which meet the adjusted 5% genome threshold. Genomic position (chromosome and base-pair location), allelic polymorphism (A and B), minor allele frequency (MAF), effect (b) and its standard error (s.e), significance ($-\log_{10}(P)$), as well as overlapping gene or nearest gene of SNPs showing genome wide significance. Coordinates based on *S.salar* Ssal_3.1 genome assembly.

Chr.	bp	A	B	MAF	b	s.e	$-\log_{10}(P)$	Trait	Group	Gene	Nearest Gene
9	21914899	G	T	0.45	0.28	0.055	6.60	length	RAS	<i>pgfb</i>	
10	45990818	T	C	0.50	-0.02	0.004	6.23	K	RAS	<i>lrp8</i>	
10	47402831	A	C	0.50	-0.02	0.004	6.17	K	RAS	<i>LOC106560530</i>	
12	19883879	G	C	0.42	0.32	0.062	6.41	length	RAS	<i>LOC123725452</i>	
22	334834	C	T	0.12	0.03	0.006	7.12	K	RAS		<i>LOC106582623</i>
22	2496554	G	T	0.11	0.03	0.006	6.59	K	RAS	<i>LOC106582632</i>	
22	5723842	T	G	0.16	0.03	0.005	7.23	K	RAS		<i>LOC106582673</i>
22	6138261	G	T	0.19	0.02	0.005	6.15	K	RAS	<i>ppp1r16b</i>	

Appendix A

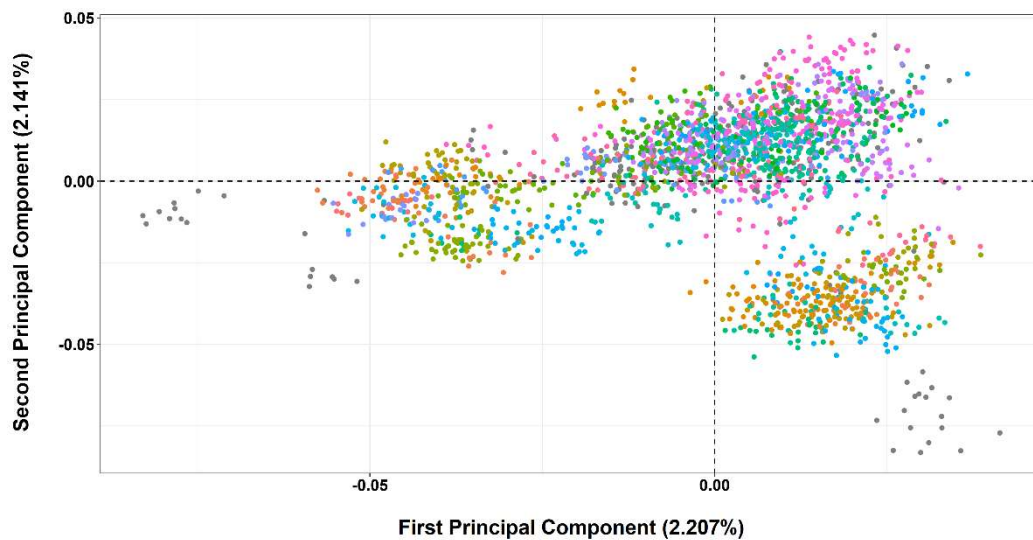
A. 11 From GWAS, additional information of SNPs found in significant association with whole-body weight (WBW), length, and condition factor (K) which meet the adjusted 5% chromosome threshold. Genomic position (chromosome and base-pair location), allelic polymorphism (A and B), minor allele frequency (MAF), effect (b) and its standard error (s.e), significance ($-\log_{10}(P)$), as well as overlapping gene or nearest gene of SNPs showing genome wide significance. Coordinates based on *S.salar* Ssal_3.1 genome assembly.

Chr.	bp	A	B	MAF	b	s.e	$-\log_{10}(P)$	Trait	group	Gene	Nearest Gene
6	55995298	A	C	0.46	-3.60	0.85	4.63	WBW	RAS	lgals8a	
6	56004795	A	G	0.45	-3.87	0.88	4.98	WBW	RAS		LOC106607667
6	56008205	A	G	0.37	-0.30	0.06	5.55	length	RAS	LOC106607667	
6	56008205	A	G	0.37	-4.57	0.94	5.91	WBW	RAS	LOC106607668	
8	13005872	C	A	0.47	-4.02	0.87	5.47	WBW	RAS	LOC106610178	
8	13694966	T	C	0.25	4.38	1.02	4.76	WBW	RAS		LOC106610225
9	21914899	G	T	0.45	0.28	0.05	6.60	length	RAS	pgfb	
9	22226040	G	C	0.42	-0.26	0.06	5.50	length	RAS	ENSSSAG00000008095	
9	22481480	C	T	0.39	-0.23	0.06	4.62	length	RAS		LOC106611090
9	94673165	C	T	0.36	-0.39	0.09	5.01	length	loch		LOC123744807
9	94766724	A	G	0.28	-0.31	0.07	5.37	length	RAS	tsc22d3	
9	96767787	C	T	0.28	-7.00	1.56	5.14	WBW	loch	ENSSSAG000000083320	
9	96782701	A	G	0.18	-0.47	0.11	4.73	length	loch	ENSSSAG000000095566	
9	96797435	T	G	0.18	-0.48	0.11	5.13	length	loch	ENSSSAG000000083320	
9	96811937	A	G	0.18	-7.87	1.80	4.92	WBW	loch	ENSSSAG000000097723	
9	96811937	A	G	0.18	-0.50	0.11	5.32	length	loch	ENSSSAG000000097723	
10	16843445	C	A	0.17	-0.03	0.01	4.82	K	loch	LOC106613633	
10	21376501	G	A	0.09	0.64	0.14	5.10	length	loch	ENSSSAG00000113780	
10	24437130	C	G	0.11	0.60	0.13	5.34	length	loch	LOC106613838	
10	24454770	G	A	0.24	0.42	0.09	5.10	length	loch	LOC106613840	
10	30476258	G	T	0.22	-0.03	0.01	4.76	K	loch		prdx6
10	40854514	T	C	0.11	0.61	0.13	5.50	length	loch	si:ch211-191a24.3	
10	45990818	T	C	0.50	-0.02	0.00	6.23	K	RAS	lrp8	
10	47398720	G	A	0.50	-0.02	0.00	6.02	K	RAS	LOC106560530	
10	47402831	A	C	0.50	-0.02	0.00	6.17	K	RAS	LOC106560530	
10	68374806	G	A	0.30	0.27	0.06	4.81	length	RAS	ENSSSAG00000104515	
12	19883879	G	C	0.42	4.32	0.92	5.62	WBW	RAS	LOC123725452	
12	19883879	G	C	0.42	0.32	0.06	6.41	length	RAS	LOC123725452	
12	19899766	A	G	0.43	4.10	0.90	5.25	WBW	RAS	LOC123725452	
12	19899766	A	G	0.43	0.30	0.06	6.04	length	RAS	LOC123725452	
13	40473884	G	T	0.13	0.03	0.01	4.69	K	loch	LOC106567100	
13	40474212	C	T	0.13	0.04	0.01	4.77	K	loch	LOC106567100	
13	40475549	T	G	0.13	0.04	0.01	4.88	K	loch	LOC106567100	
13	40483278	G	A	0.14	0.04	0.01	4.81	K	loch	LOC106567100	
13	40484059	C	A	0.13	0.04	0.01	4.73	K	loch	LOC106567100	
15	62331139	A	G	0.38	-3.64	0.83	4.90	WBW	RAS	LOC106571856	
15	62332972	A	G	0.38	-3.70	0.83	5.04	WBW	RAS		LOC106571856
16	43224956	A	G	0.32	-3.85	0.91	4.62	WBW	RAS	LOC106573912	
17	13200053	T	C	0.07	-0.03	0.01	4.74	K	RAS		LOC106575239
19	18280978	T	C	0.51	-3.60	0.84	4.77	WBW	RAS		rab18a
19	49828526	A	G	0.16	5.21	1.22	4.74	WBW	RAS		LOC106579037

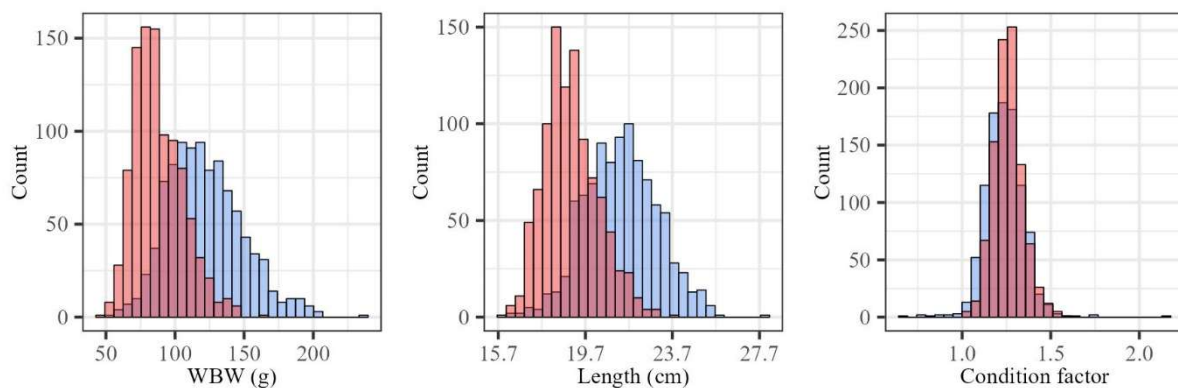
Appendix A

22	334834	C	T	0.12	6.56	1.34	6.04	WBW	RAS		LOC106582623
22	334834	C	T	0.12	0.03	0.01	7.12	K	RAS		LOC106582623
22	2496554	G	T	0.11	6.42	1.34	5.81	WBW	RAS	LOC106582632	
22	2496554	G	T	0.11	0.03	0.01	6.59	K	RAS	LOC106582632	
22	5723842	T	G	0.16	5.15	1.16	5.07	WBW	RAS		LOC106582673
22	5723842	T	G	0.16	0.03	0.01	7.23	K	RAS		LOC106582673
22	6138261	G	T	0.19	0.02	0.00	6.15	K	RAS	ppp1r16b	
22	6163393	A	G	0.35	0.02	0.00	4.99	K	RAS	ppp1r16b	
22	7146753	G	A	0.28	0.02	0.00	5.09	K	RAS	LOC106582690	
22	7824331	C	T	0.15	0.03	0.01	5.48	K	RAS	LOC106582707	
24	15406969	C	T	0.38	-0.02	0.00	4.86	K	RAS		trnas-uga
24	15418645	C	T	0.38	-0.02	0.00	5.14	K	RAS		trnas-uga

A. 12 Principal component analysis of genomic relationship matrix (GRM) for the parental (grey dots) and offspring genotypes. Families identified via pedigree reconstruction in the offspring population are indicated by different colouration of dots.

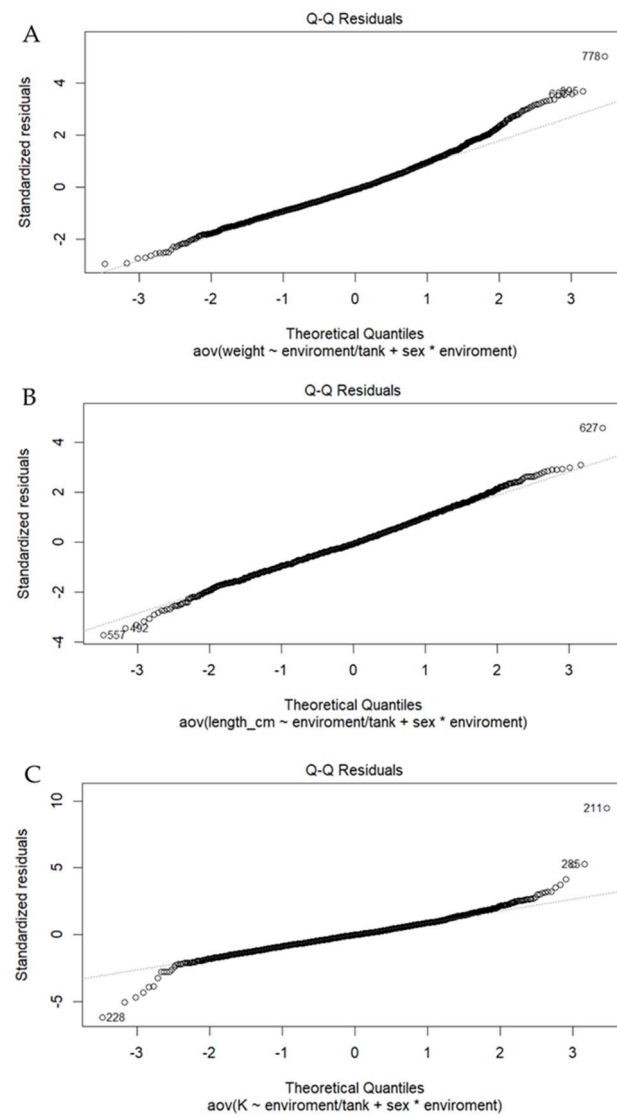


A. 13 Histogram of whole-body weight (WBW) (g), length (cm) and condition factor, split between RAS (red) and loch (blue) populations.



Appendix A

A. 14 Q-q plots for A) whole-body weight (g), B) length (cm) and C) condition factor.



Appendix B.

B.1 Sampling, treatment and phenotypic data.

SampleID	Sampling point	Tank	Photoperiod	Feed-regime	Temperature	Weight (g)	Length (cm)
61	BL	A4	LL	CF	LT	19.27	11.9
62	BL	A4	LL	CF	LT	25.97	12.9
63	BL	A5	LL	CF	LT	13.28	10.6
64	BL	A5	LL	CF	LT	24.59	12.8
65	BL	A6	LL	CF	LT	21.75	12.3
66	BL	A6	LL	CF	LT	27.5	13.6
67	800	A1	LL	CF	LT	58.12	16.5
68	800	A1	LL	CF	LT	45.05	15.7
69	800	A2	LL	CF	LT	48.36	15.7
70	800	A2	LL	CF	LT	52.32	16.3
71	800	A3	LL	CF	LT	71.8	18.1
72	800	A3	LL	CF	LT	42.2	16.1
73	800	A4	LL	DF	LT	62.46	16.9
74	800	A4	LL	DF	LT	61.7	17.4
75	800	A5	LL	DF	LT	56.1	17.1
76	800	A5	LL	DF	LT	65.91	17.5
77	800	A6	LL	DF	LT	57.63	16.6
78	800	A6	LL	DF	LT	44.99	15.4
79	800	A7	LND	DF	LT	59.32	16.7
80	800	A7	LND	DF	LT	58.6	16.8
81	800	A8	LND	DF	LT	52.6	16.2
82	800	A8	LND	DF	LT	56.79	17.2
83	800	A9	LND	DF	LT	60.63	17.1
84	800	A9	LND	DF	LT	36.26	14.6
85	800	B1	LL	DF	HT	65.9	18.1
86	800	B1	LL	DF	HT	44.9	16.1
87	800	B2	LL	DF	HT	78	18.7
88	800	B2	LL	DF	HT	95.8	20.3
89	800	B3	LL	DF	HT	64.81	14.5
90	800	B3	LL	DF	HT	84.51	20.4
91	800	B4	LL	CF	HT	79.1	19
92	800	B4	LL	CF	HT	63.1	17.8
93	800	B5	LL	CF	HT	73.7	18.5
94	800	B5	LL	CF	HT	88.1	19.3
95	800	B6	LL	CF	HT	58.1	17.5
96	800	B6	LL	CF	HT	61.3	17.2
97	800	B10	LND	DF	HT	51.8	16.5
98	800	B10	LND	DF	HT	62	17.5
99	800	B11	LND	DF	HT	49.1	16.3
100	800	B11	LND	DF	HT	93.1	19.7
101	800	B12	LND	DF	HT	42.9	15.3
102	800	B12	LND	DF	HT	50.9	16.9

Appendix B

B.2 RRBS sequencing information. * indicates sample was removed for analysis.

SampleID	Treatment	Timepoint	Reads (pre-filtering)	Reads (pass-filtering)	Duplication rate (%)	Sequence pairs analysed in total	% unique mapping	Cytosines analysed	% C's in CpG	Library size (unmethylatedC)	Library size (methylatedC)	% Methylated	% of methylated C's in CpGs
61	LL.CF.LT	Baseline	48808670	24575642	54.26	12287821	54.28	2.57E+08	18.16	33511950	13221546	71.71	96.07
62	LL.CF.LT	Baseline	50540032	26714420	60.27	13357210	54.9	2.99E+08	17.13	36397336	14887616	70.97	95.55
63	LL.CF.LT	Baseline	33542170	18923566	58.25	9461783	55.54	2.13E+08	17.16	26081480	10508866	71.28	95.52
64	LL.CF.LT	Baseline	48067808	25965460	54.16	12982730	53.53	2.86E+08	18.1	37242772	14536729	71.93	96.19
65	LL.CF.LT	Baseline	85156116	36062864	66.45	18031432	54.35	3.93E+08	17.92	50825394	19596414	72.17	96.71
66	LL.CF.LT	Baseline	37038682	23188440	49.82	11594220	55.58	2.63E+08	17.72	33452211	13202364	71.7	96.37
67	LL.DF.LT	800dd	38887604	18871606	63.28	9435803	52.98	2.01E+08	17.68	25380213	10103038	71.53	95.66
68	LL.DF.LT	800dd	42224768	25873708	50.18	12936854	56.23	2.95E+08	17.62	36474167	15445661	70.25	96.29
69	LL.DF.LT	800dd	57083416	28317030	61.95	14158515	53.66	3.07E+08	18.14	40543198	15157967	72.79	96.49
70	LL.DF.LT	800dd	56549932	35770974	51.5	17885487	54.54	3.98E+08	17.83	50374356	20491173	71.08	96.04
71*	LL.DF.LT	800dd	52298166	31728204	51.62	15864102	54.66	3.57E+08	18.15	44881363	19920188	69.26	96.4
72*	LL.DF.LT	800dd	120931530	42528278	68.77	21264139	51.67	4.36E+08	18.16	55339834	23818244	69.91	96.65
73	LL.CF.LT	800dd	64238066	41029896	52.23	20514948	55.59	7.08E+08	17.59	59511959	24495880	70.84	#DIV/0!
74	LL.CF.LT	800dd	86410300	35822822	66.73	17911411	53.87	3.85E+08	18.01	49940894	19437818	71.98	96.67
75	LL.CF.LT	800dd	57211260	25806746	56.67	12903373	55.01	2.79E+08	17.96	36134073	14017275	72.05	96.1
76	LL.CF.LT	800dd	73778252	43182278	37.65	21591139	19.55	1.65E+08	18.59	22399542	8201194	73.2	93.17
77	LL.CF.LT	800dd	89191994	36807720	67.54	18403860	56.34	4.19E+08	18.04	54738531	20820331	72.44	96.75
78	LL.CF.LT	800dd	84244380	29061798	66.73	14530899	53.73	3.26E+08	17.98	42713787	15854035	72.93	96.77
79	LND.DF.LT	800dd	45053100	17293796	55.04	8646898	49.86	1.7E+08	18.19	22199405	8784399	71.65	96.17
80	LND.DF.LT	800dd	61135474	36201218	54.36	18100609	51.46	3.85E+08	18	50058146	19199133	72.28	96.16
81	LND.DF.LT	800dd	54914212	24781776	56.1	12390888	51.37	2.55E+08	18.24	33564554	13031037	72.03	96.17
82	LND.DF.LT	800dd	96179378	39029330	65.03	19514665	54.53	4.27E+08	17.89	54679928	21644529	71.64	96.68
83	LND.DF.LT	800dd	88874066	35009054	61.3	17504527	51.82	3.68E+08	18.1	47958986	18679504	71.97	96.17
84	LND.DF.LT	800dd	52642216	19233556	58.34	9616778	50.97	1.96E+08	18.21	25484028	10235798	71.34	96.17
85	LL.CF.HT	800dd	37261402	13589902	50.75	6794951	53.62	1.47E+08	18.36	19420691	7656958	71.72	96.14
86*	LL.CF.HT	800dd	65609716	45549228	50.02	22774614	55.9	5.29E+08	17.35	65641963	26068257	71.58	96.25
87*	LL.CF.HT	800dd	3444930	271268	61.27	135634	13.67	754710	17.89	97495	37493	72.22	93.44
88	LL.CF.HT	800dd	35595544	18464774	60.14	9232387	52.86	1.96E+08	17.6	23851885	10630423	69.17	95.57
89	LL.CF.HT	800dd	36944098	17398168	61.63	8699084	55.11	1.95E+08	17.63	24632026	9685974	71.78	95.67
90	LL.CF.HT	800dd	45865912	26477584	59.15	13238792	55.49	3.06E+08	17.28	37564608	15365049	70.97	95.54
91	LL.DF.HT	800dd	50699364	28882164	55.05	14441082	54.99	3.19E+08	18.09	41893178	15724689	72.71	96.18
92	LL.DF.HT	800dd	42814672	25631680	52.36	12815840	52.64	2.74E+08	17.75	34142280	14496592	70.2	95.96
93	LL.DF.HT	800dd	54334484	22884778	60.4	11442389	49.71	2.25E+08	18.06	28192589	12453409	69.36	96.04
94	LL.DF.HT	800dd	59145862	30832386	57.77	15416193	52.09	3.18E+08	17.62	39416827	16576895	70.4	95.97
95	LL.DF.HT	800dd	43800236	14543364	55.32	7271682	49.99	1.48E+08	18.58	19342476	8152412	70.35	96.24
96	LL.DF.HT	800dd	55261594	22591626	52.27	11295813	53.41	2.35E+08	18.04	29289068	13062278	69.16	95.95
97	LND.DF.HT	800dd	38103032	13133654	61.91	6566827	50.76	1.3E+08	18.21	16902041	6717201	71.56	96.15
98*	LND.DF.HT	800dd	53570140	23646232	63.64	11823116	51.69	2.4E+08	18.5	32397766	12090158	72.82	96.54
99	LND.DF.HT	800dd	69109674	29767908	65.4	14883954	52.02	3.11E+08	18.22	40586783	16058928	71.65	96.47
100	LND.DF.HT	800dd	54184246	21667894	65.17	10833947	51.68	2.24E+08	18.42	29943540	11257630	72.68	96.54
101	LND.DF.HT	800dd	56899230	23214466	66.24	11607233	53.12	2.44E+08	18.49	33104844	12079706	73.27	96.59
102	LND.DF.HT	800dd	45456550	18844030	62.3	9422015	54.51	2.07E+08	18.01	27056526	10175253	72.67	96.46

Appendix B

B. 3 Average and standard deviation (s.d) increase in whole-body weight (WBW) (g) and length (cm) in the high (HT) and low (LT) temperature groups from the baseline, as well as the difference between these. HT is high temperature and LT is low temperature.

	Change since baseline		
	HT	LT	Difference
WBW (g) (s.d)	+ 33.86 (2.78)	+ 44.05 (4.23)	10.19
Length (cm) (s.d)	+ 4.30 (0.22)	+ 5.44 (0.35)	1.14

B. 4 Output of the chi-square goodness of fit test when the number of DMC genomic annotation compared to the background set proportions. In this LT is low temperature, HT is high temperature, DF is feeding in the perceived day, CF is continuous feeding LND is light dark photoperiod, and LL is continuous light

<i>Treatment</i>	X-square	DF	P-value
<i>LNF.LT</i>	2.44	6	0.87
<i>DF.LT</i>	3.96	6	0.68
<i>LL.DF.LT</i>	5.91	6	0.43
<i>LND.DF.LT</i>	7.74	6	0.26
<i>LNF.HT</i>	5.83	6	0.44
<i>DF.HT</i>	2.21	6	0.90
<i>LL.DF.HT</i>	6.77	6	0.34
<i>LND.DF.HT</i>	3.27	6	0.77

B. 5 Output of chi square test for association between hyper and hypo methylated putative DMC annotations within each treatment group. * Indicates exonic DMC category was omitted as was zero for both hyper and hyper methylation cytosines. In this LT is low temperature, HT is high temperature, DF is feeding in the perceived day, CF is continuous feeding LND is light dark photoperiod, and LL is continuous light.

<i>Treatment</i>	X-square	DF	P-value
<i>LND.LT</i>	9.57	6	0.14
<i>DF.LT</i>	9.85	6	0.13
<i>LL.DF.LT</i>	3.04	5*	0.69
<i>LND.DF.LT</i>	6.09	6	0.41
<i>LND.HT</i>	4.50	6	0.60
<i>DF.HT</i>	6.01	6	0.42
<i>LL.DF.HT</i>	4.96	6	0.55
<i>LND.DF.HT</i>	9.27	6	0.16

Appendix B

B. 6 RNA sequencing information. * indicates sample was removed for analysis. In this LT is low temperature, HT is high temperature, DF is feeding in the perceived day, CF is continuous feeding LND is light dark photoperiod, and LL is continuous light.

<i>Sampled</i>	<i>Treatment</i>	<i>Timepoint</i>	<i>pre filtering reads</i>	<i>post filtering reads</i>	<i>Reads retained (%)</i>	<i>duplication rate (%)</i>
61	LL.CF.LT	Baseline	37457626	33589866	89.67	32.74
62	LL.CF.LT	Baseline	53845282	48023720	89.19	21.82
63	LL.CF.LT	Baseline	45448358	41007170	90.23	15.4
64	LL.CF.LT	Baseline	41063928	36960330	90.01	14.3
65	LL.CF.LT	Baseline	56184850	50290594	89.51	22.59
66	LL.CF.LT	Baseline	53555400	47612024	88.9	20.13
67	LL.DF.LT	800dd	56536800	50557872	89.42	39.7
68	LL.DF.LT	800dd	37876370	34173386	90.22	22.06
69	LL.DF.LT	800dd	52581542	46965382	89.32	28.86
70	LL.DF.LT	800dd	60555010	54265284	89.61	31.11
71*	LL.DF.LT	800dd	50772556	45501212	89.62	28.39
72*	LL.DF.LT	800dd	51166080	45493388	88.91	52.59
73	LL.CF.LT	800dd	50026866	44838576	89.63	26.01
74	LL.CF.LT	800dd	54695062	48741354	89.11	27.21
75	LL.CF.LT	800dd	51596200	46409928	89.95	28.51
76	LL.CF.LT	800dd	56635416	50701134	89.52	24.15
77	LL.CF.LT	800dd	60075512	53721024	89.42	34.73
78	LL.CF.LT	800dd	42437736	38253790	90.14	21.19
79	LND.DF.LT	800dd	53751898	48186232	89.65	33.36
80	LND.DF.LT	800dd	54216388	48464944	89.39	32.73
81	LND.DF.LT	800dd	57838820	51891484	89.72	37.32
82	LND.DF.LT	800dd	38777046	35038502	90.36	21.66
83	LND.DF.LT	800dd	71012712	63689606	89.69	40.55
84	LND.DF.LT	800dd	59051766	52929530	89.63	32.3
85	LL.CF.HT	800dd	50526626	44955676	88.97	19.22
86*	LL.CF.HT	800dd	64321502	56862256	88.4	64.7
87*	LL.CF.HT	800dd	55142800	49377760	89.55	50.65
88	LL.CF.HT	800dd	62423196	56012780	89.73	27.93
89	LL.CF.HT	800dd	47139882	42042872	89.19	21.85
90	LL.CF.HT	800dd	52669600	47085970	89.4	21.68
91	LL.DF.HT	800dd	62930396	56199438	89.3	23.5
92	LL.DF.HT	800dd	68517812	61139630	89.23	25.13
93	LL.DF.HT	800dd	54709914	48928166	89.43	20.96
94	LL.DF.HT	800dd	58344512	51836372	88.85	22.89
95	LL.DF.HT	800dd	57336676	51278638	89.43	47.68
96	LL.DF.HT	800dd	59086578	53008370	89.71	42.15
97	LND.DF.HT	800dd	49829414	44546446	89.4	35.57
98*	LND.DF.HT	800dd	40569700	36060704	88.89	63.26
99	LND.DF.HT	800dd	63054442	56343068	89.36	46.69
100	LND.DF.HT	800dd	61056498	54418702	89.13	37.38
101	LND.DF.HT	800dd	57771086	51795474	89.66	40.32
102	LND.DF.HT	800dd	62659094	56115302	89.56	37.25

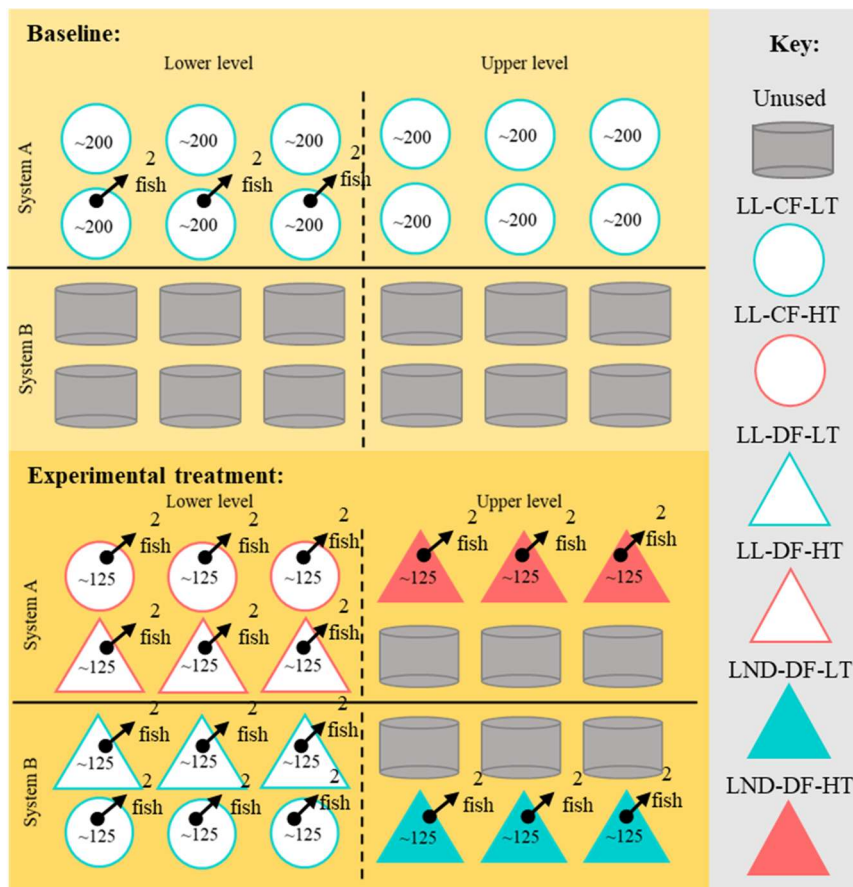
Appendix B

B. 7 GO terms associated with DEG in high and low temperature groups for treatments, as well as DEG_{time} at high temperature. In this LT is low temperature, HT is high temperature, DF is feeding in the perceived day, CF is continuous feeding LND is light dark photoperiod, and LL is continuous light.

		Gene ID	Change	GO	Accession	GO terms
LT	LND	ENSSSAG000000109033	down-regulated			
LT	LND	ENSSSAG00000020579	up-regulated			
LT	LND	ENSSSAG000000066674	down-regulated			
LT	LND	ENSSSAG00000008052	up-regulated			
LT	LND	ENSSSAG000000094667	up-regulated			
LT	LND,DF	ENSSSAG000000043460	down-regulated	BP	GO:0070588	calcium ion transmembrane transport
				MF	GO:0005245	voltage-gated calcium channel activity
				MF	GO:0005515	protein binding
				CC	GO:0005891	voltage-gated calcium channel complex
LT	LND,DF	ENSSSAG000000109605	down-regulated			
LT	LND,DF	ENSSSAG000000114955	down-regulated	BP	GO:0007018	microtubule-based movement
				MF	GO:0003777	microtubule motor activity
				MF	GO:0005524	ATP binding
				MF	GO:0008017	microtubule binding
LT	LND and LND,DF	ENSSSAG000000076584	up-regulated	MF	GO:0003796	lysozyme activity
HT	Time	ENSSSAG000000015649	Up-regulated			
HT		ENSSSAG000000068220	Down-regulated	CC	GO:0031012	extracellular matrix
				BP	GO:0006508	proteolysis
				MF	GO:0004222	metalloendopeptidase activity
					GO:0008237	metallopeptidase activity
					GO:0008270	zinc ion binding
HT	Time	ENSSSAG000000077769	Down-regulated	BP	GO:0006508	proteolysis
				BP	GO:0030198	extracellular matrix
				MF	GO:0004222	metalloendopeptidase activity
					GO:0008237	metallopeptidase activity
					GO:0008270	zinc ion binding
HT	Time	ENSSSAG000000055437	Down-regulated			
HT	DF	ENSSSAG000000057719	up-regulated	MF	GO:0005179	hormone activity
				CC	GO:0005576	extracellular region
HT	DF and LL,DF	ENSSSAG000000093579	up-regulated	BP	GO:0015671	oxygen transport
				MF	GO:0019825	oxygen binding
				MF	GO:0020037	heme binding
				CC	GO:0005833	hemoglobin complex
HT	DF	ENSSSAG000000061935	up-regulated	MF	GO:0008757	S-adenosylmethionine-dependent methyltransferase activity
HT	DF	ENSSSAG000000040068	up-regulated	MF	GO:0030246	Carbohydrate binding
HT	LND and LL,DF	ENSSSAG000000114681	up-regulated	MF	GO:0008757	S-adenosylmethionine-dependent methyltransferase activity
HT	LL,DF	ENSSSAG000000119855	up-regulated			

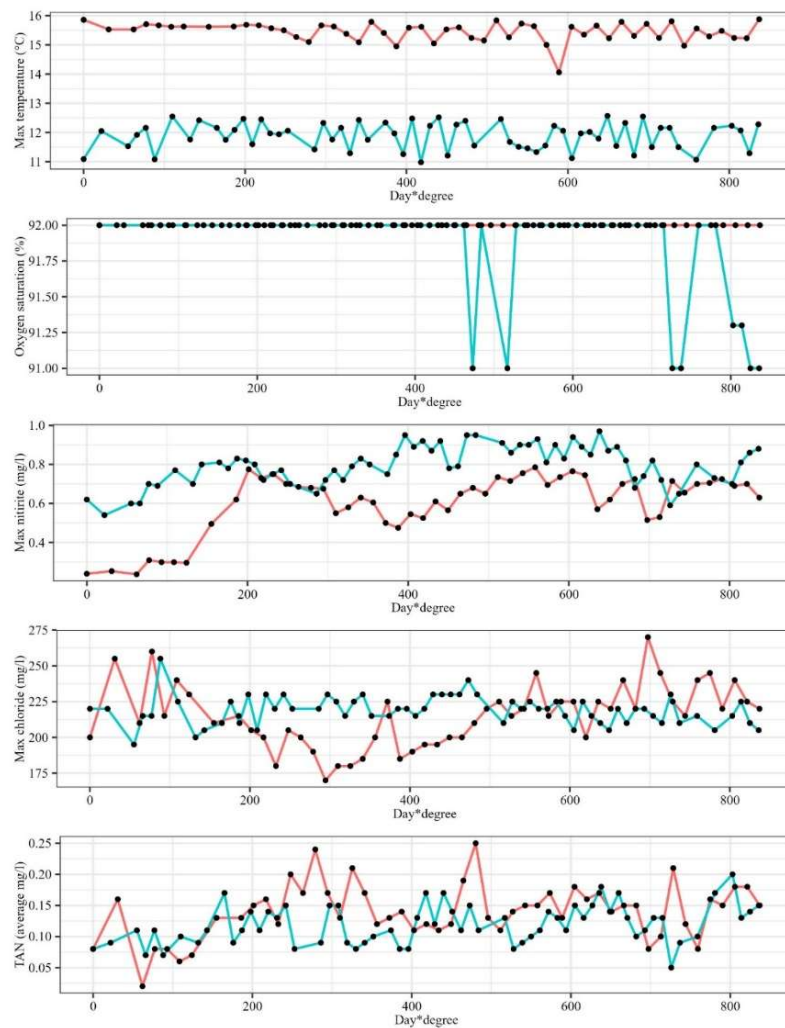
Appendix B

B. 8 Experimental design of aquarium for the experimental study. Arrows indicate the number of fish per tank sampled at both the baseline and after 800dd exposure to experimental treatments.

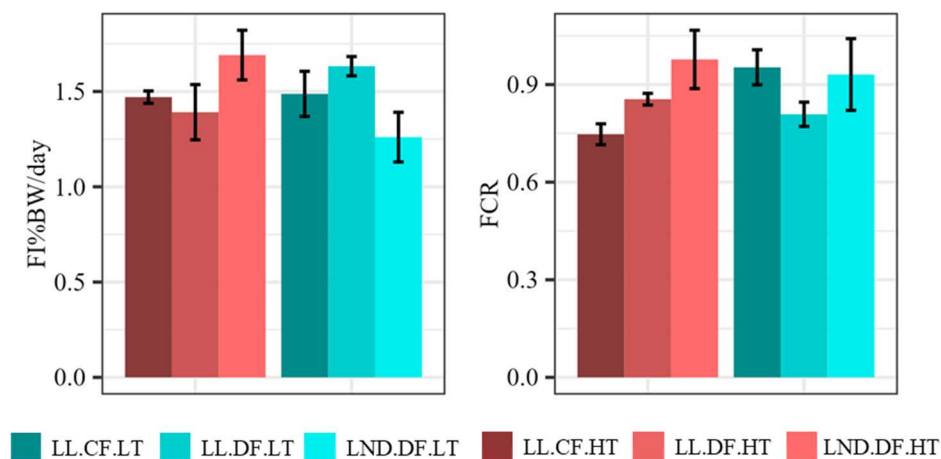


Appendix B

B. 9 Mean maximum daily temperature, oxygen saturation (%), maximum nitrate (mg/l), maximum chloride (mg/l), and total available nitrogen (TAN) (average mg/l) over the experimental period (approximately 800dd). Red line indicates system A, or the high temperature treatment, and the turquoise line indicates system B, or the low temperature treatment.

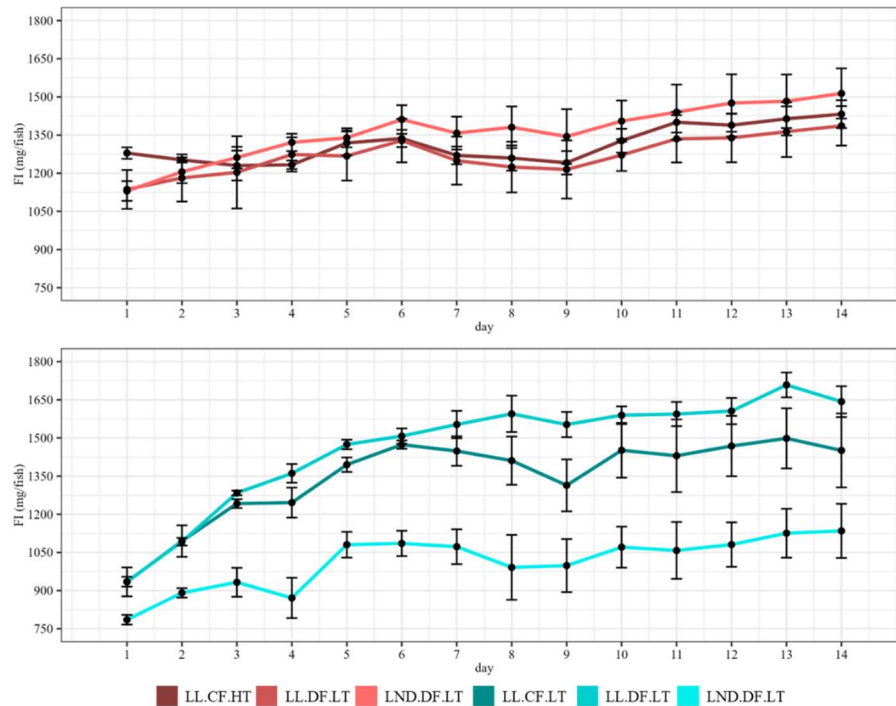


B. 10 Mean and standard error for feed intake as a percentage of body weight per day, and feed conversion ratio (FCR) across treatment groups. In this LT is low temperature, HT is high temperature, DF is feeding in the perceived day, CF is continuous feeding LND is light dark photoperiod, and LL is continuous light.

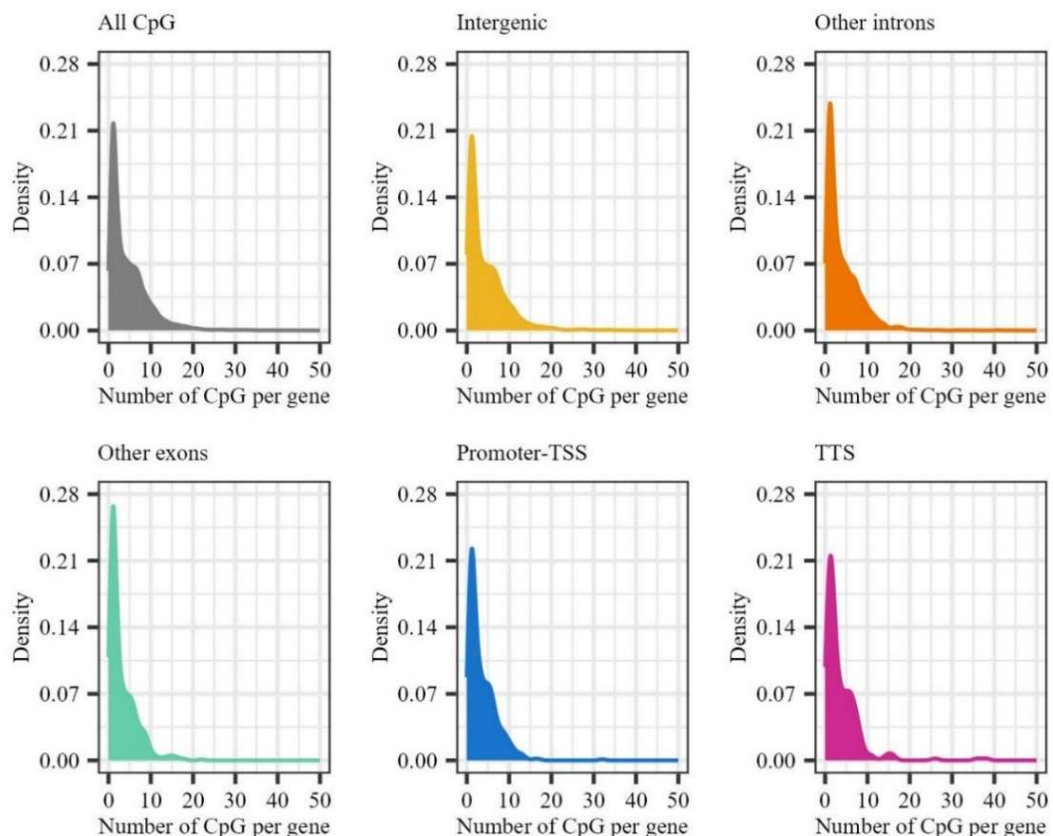


Appendix B

B. 11 Average daily feed intake in mg/fish. Average was taken across triplicate tank treatments. Error bars are standard error. In this LT is low temperature, HT is high temperature, DF is feeding in the perceived day, CF is continuous feeding LND is light dark photoperiod, and LL is continuous light

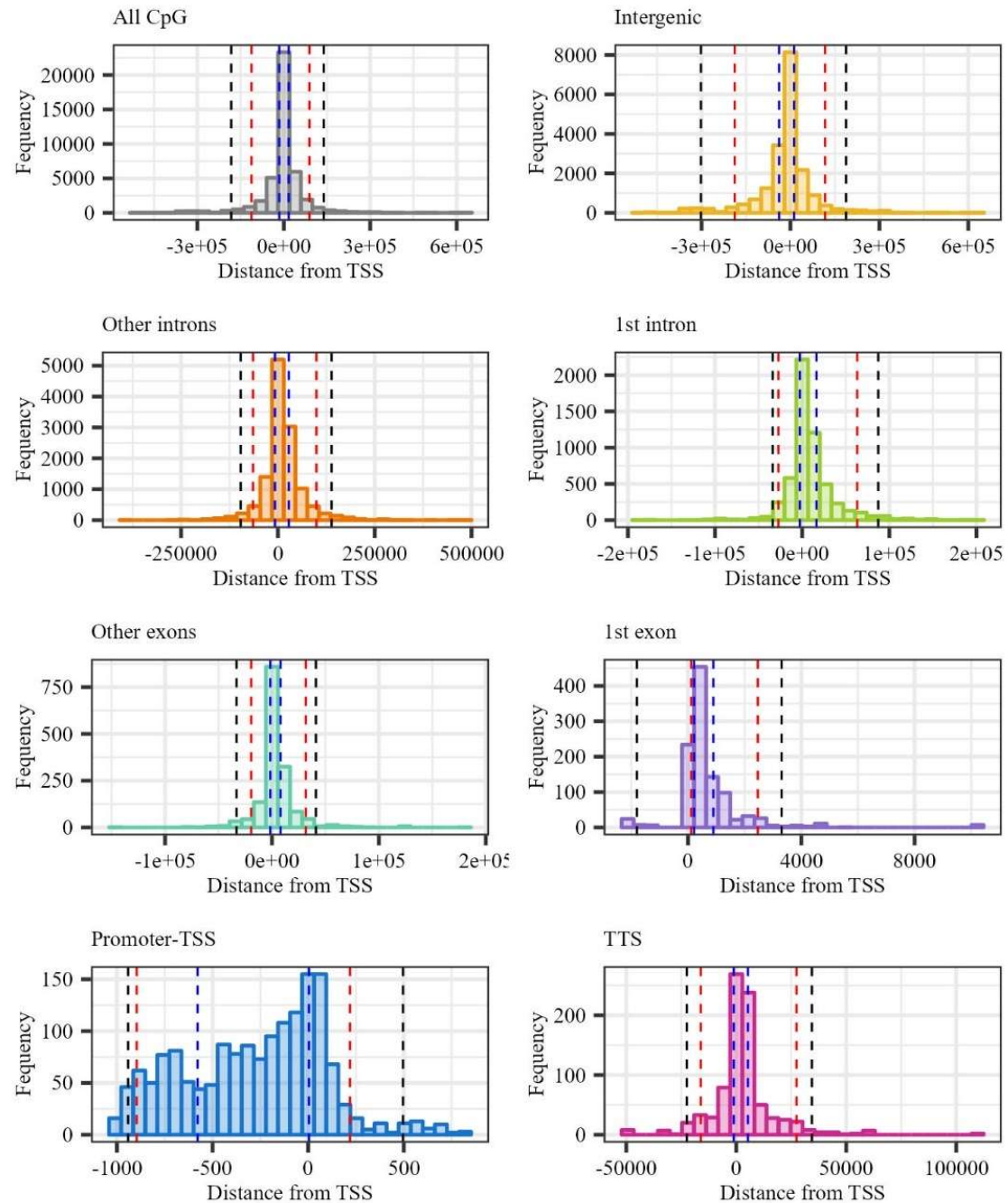


B. 12 Density plot of the number of CpG sites annotated to the same gene. 1st intron and exon omitted as all would exists in a 1t:1 relationship as only a single CPG in these categories can exists per gene. For visualisation genes ENSSSAG00000089291, ENSSSAG00000122189 and ENSSSAG00000083685 were removed as had outlier numbers of CpGs to a single gene, specifically 870, 165 and 136 respectively.



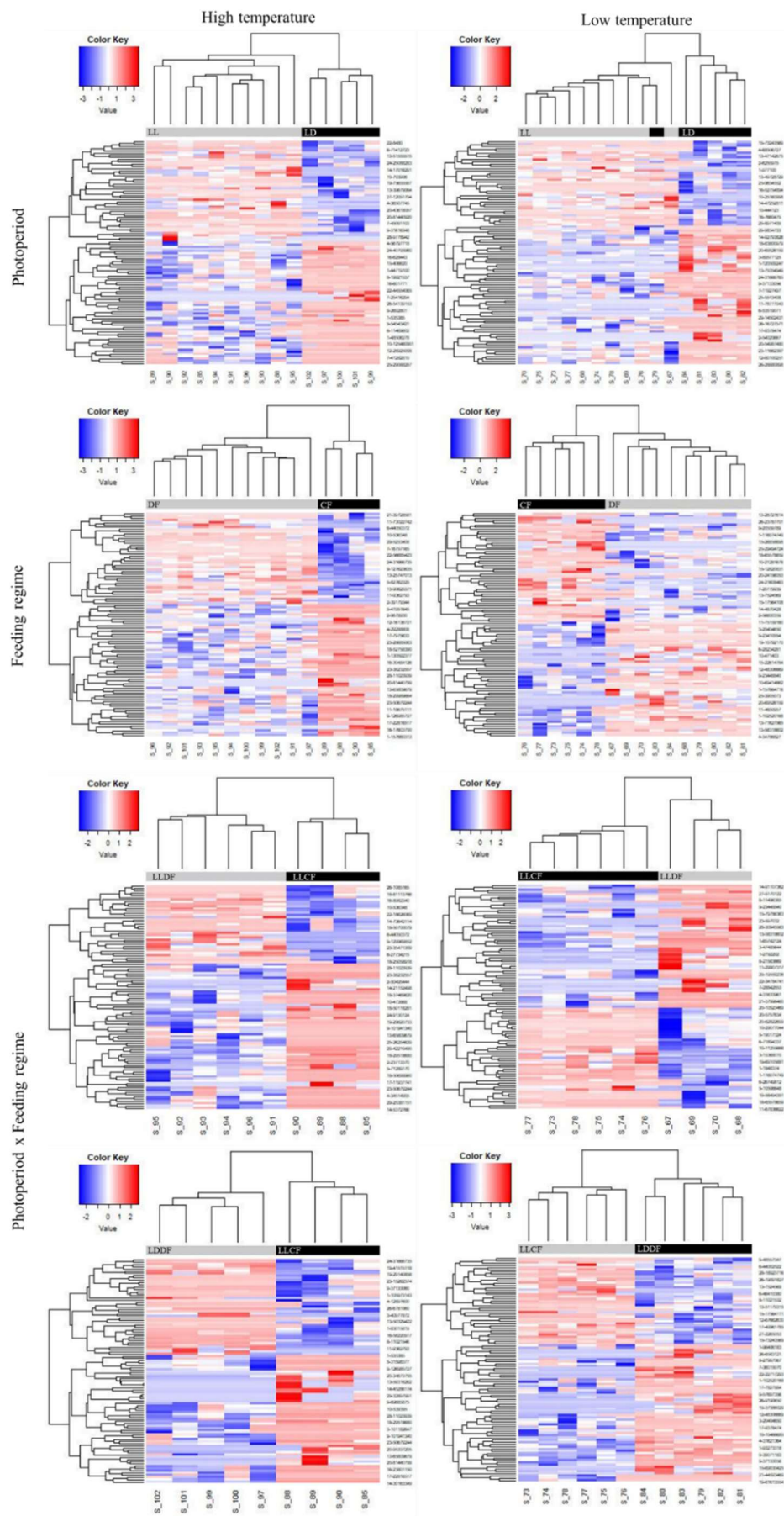
Appendix B

B. 13 Histogram of CpG sites distance from the TSS where red, blue and black lined indicated boundaries for the 50%, 90% and 95% percentiles.



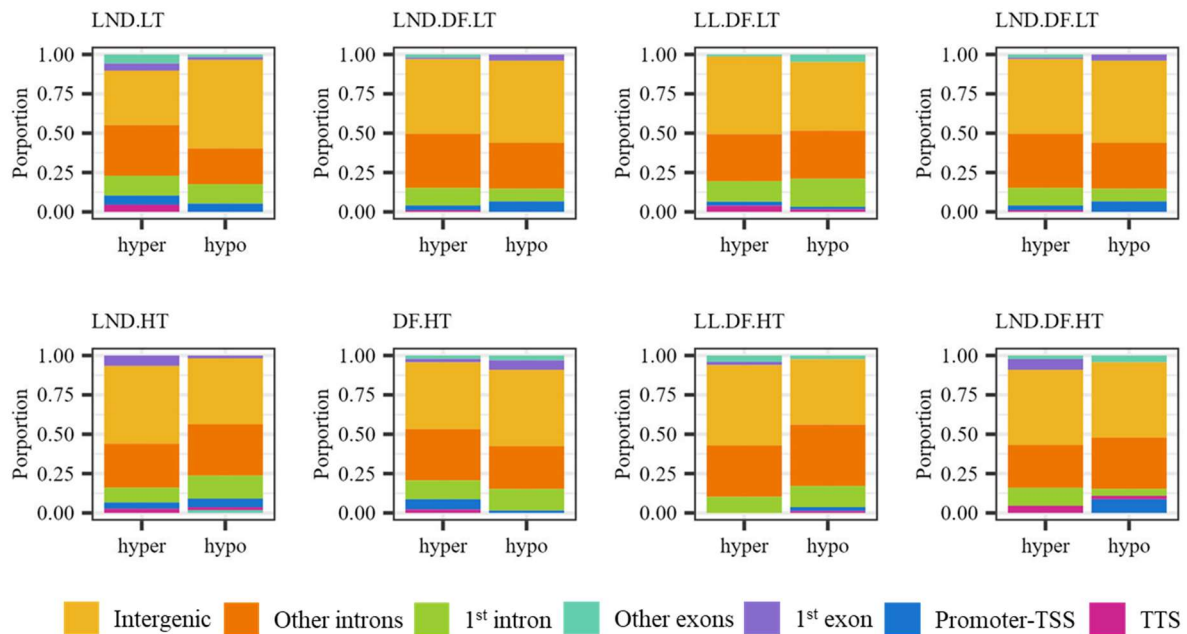
Appendix B

B.14 Heat maps of the 100 most differently methylated cytosines

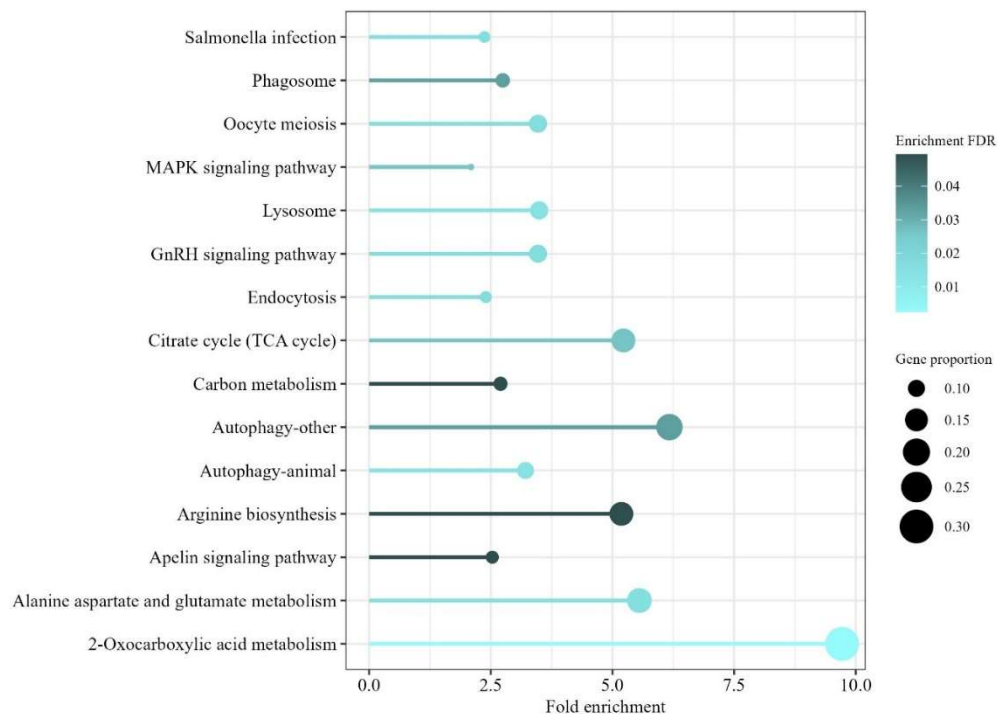


Appendix B

B. 15: Proportion of DMC annotated to different genomic features for hyper and hypo methylated relative to control conditions in each treatment group. LT is low temperature, HT high temperature, LL is continuous slight, LND is light dark cycles, CF continuous feeding, and DF is feeding in the perceived day.

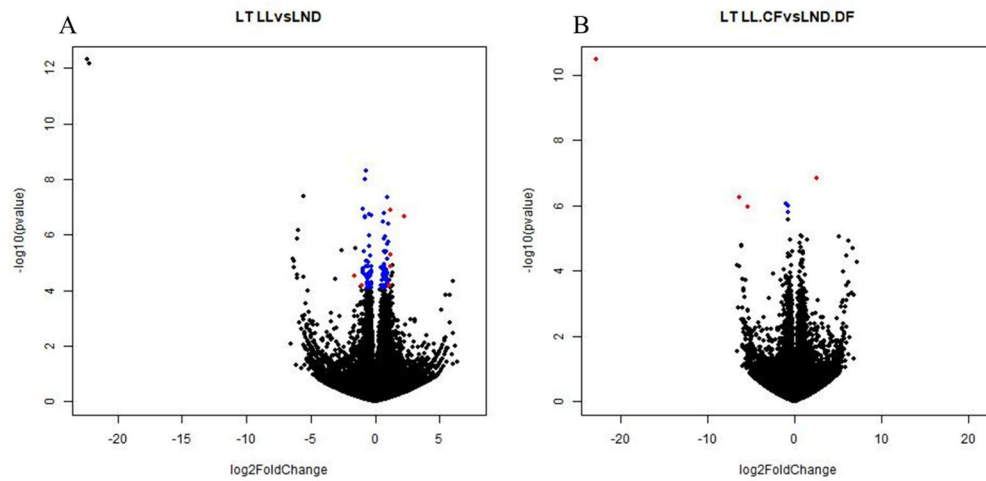


B. 16 Visualisation of KEGG enrichment for DEG down-regulated in LL.CF.LT at 800dd relative to baseline. Coloured by enrichment false discovery rate (FDR) and the size of dots represent the number DEG up-regulated in a pathway, as a proportion of all genes in that pathway.

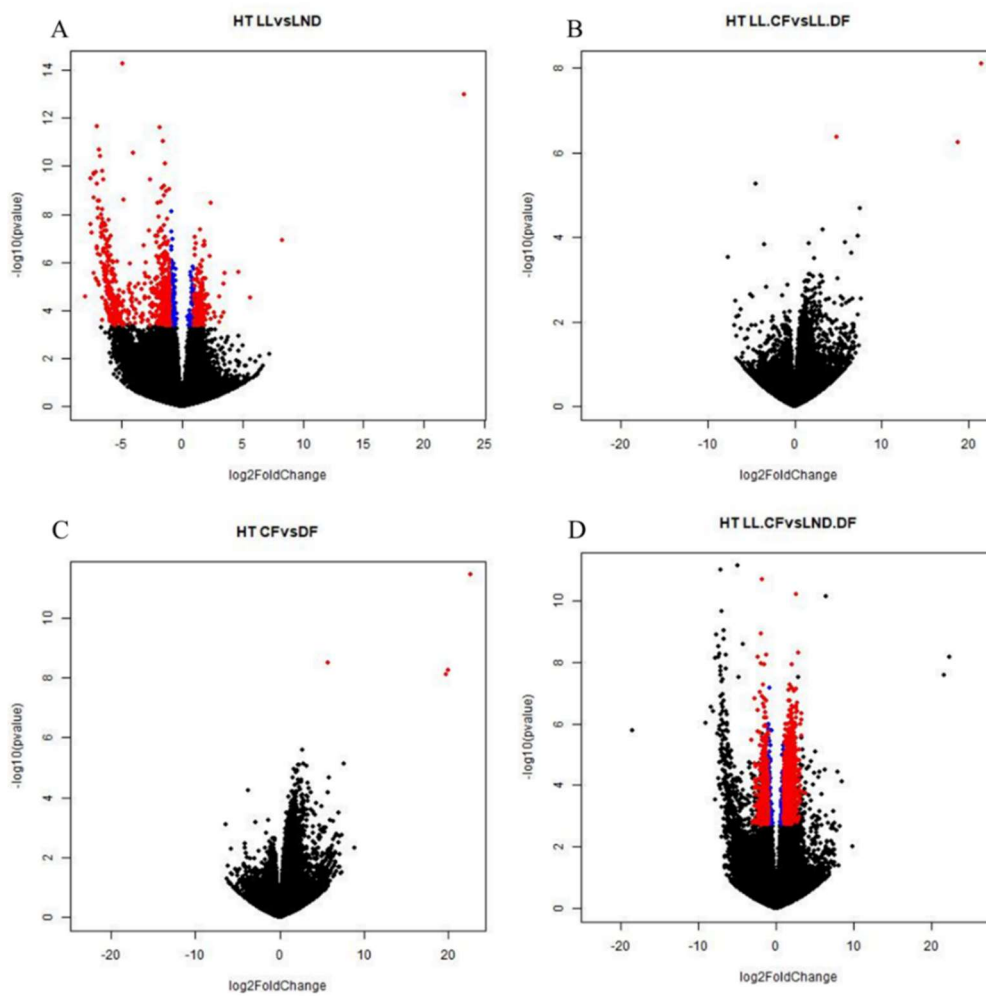


B. 17 Volcano plot of differential gene expression analysis for the low temperature treatment groups. Blue dots are those meeting p-value threshold, red dots are those meeting both p-value and logfold2 threshold. LT is low temperature, LL is continuous slight, LND is light dark cycles, CF continuous feeding, and DF is feeding in the perceived day.

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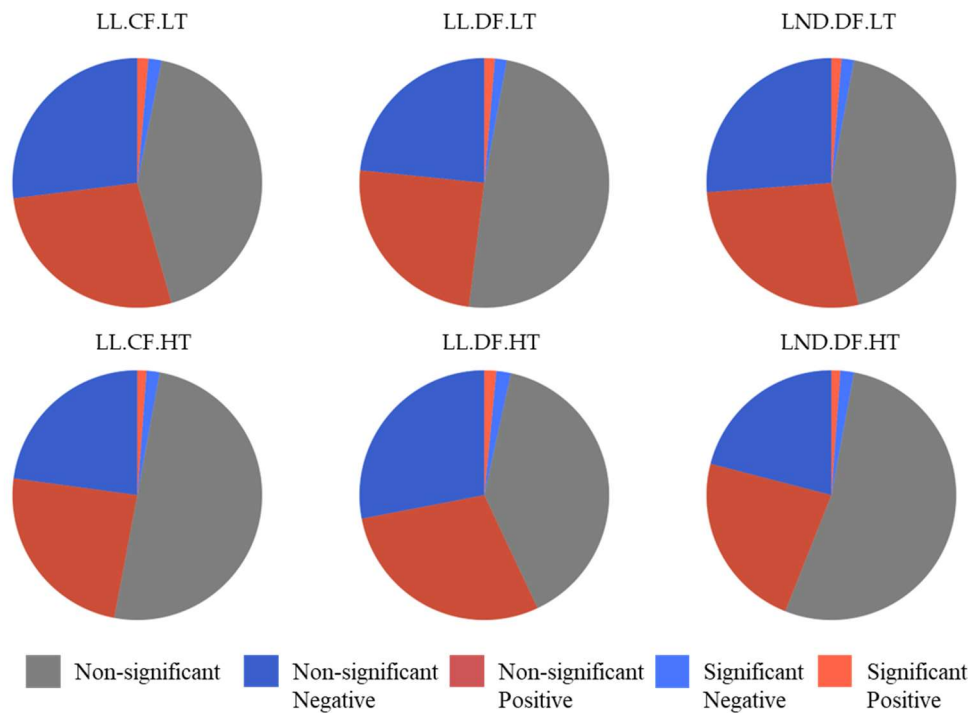


B. 18 Volcano plot of differential gene expression analysis for the high temperature treatment groups. Blue dots are those meeting p-value threshold, red dots are those meeting both p-value and logfold2 threshold. HT high temperature, LL is continuous slight, LND is light dark cycles, CF continuous feeding, and DF is feeding in the perceived day.

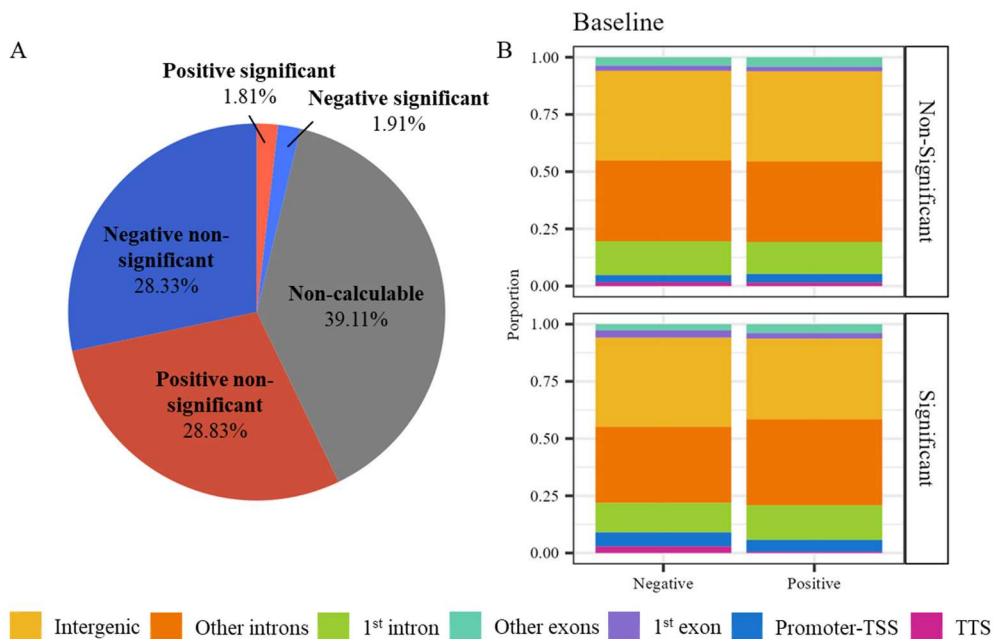


Appendix B

B. 19 Pie chart demonstrating the percentage of filtered CpG sites for baseline samples, where the proportionate methylation was in significant, non-significant or non-calculable correlation with overlapping genes normalized (FPKM) gene expression across all samples (n=37). Pearsons correlation at 5% significance. B) Genomic annotation of CpG-gene combinations in significant and non-significant correlations.

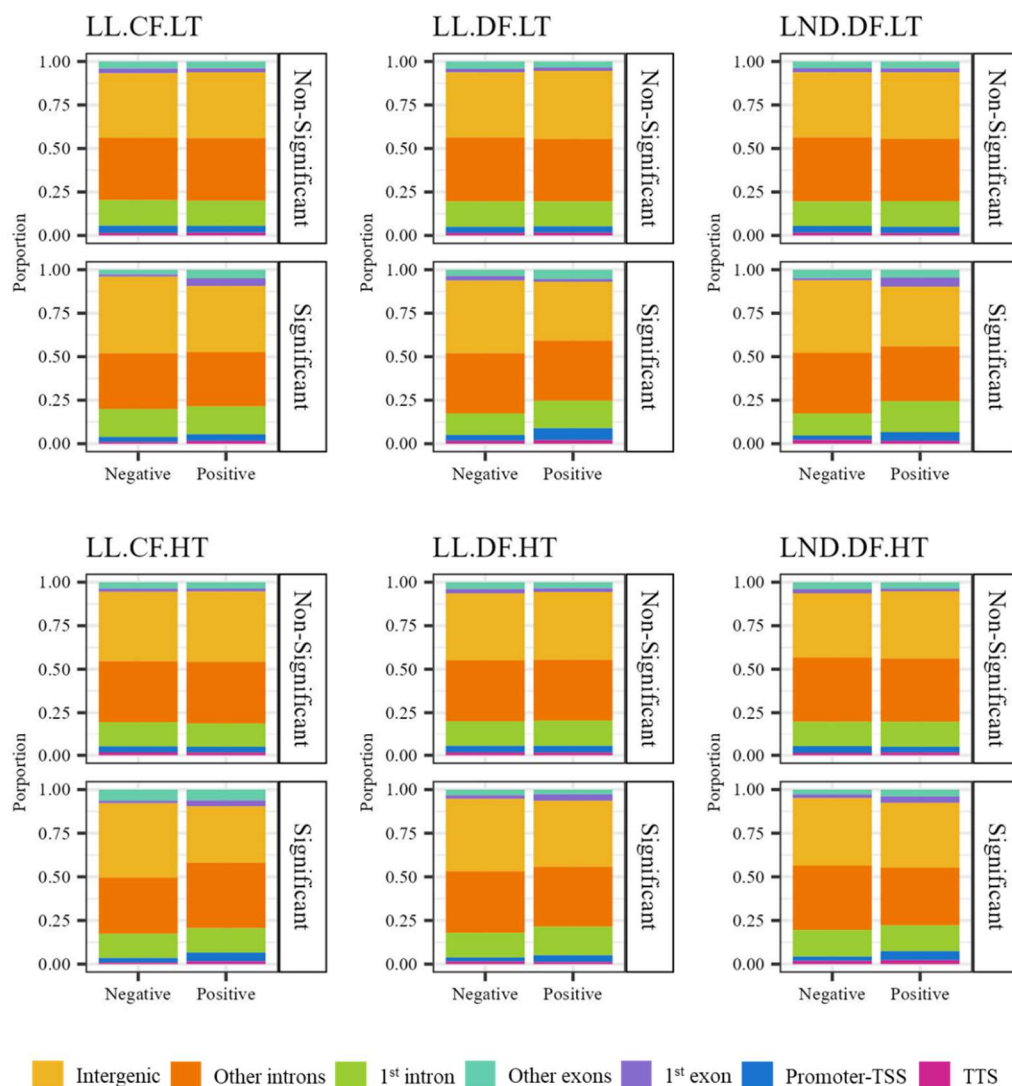


B. 20 For all filtered CpG sites, pie charts demonstrating the proportion of Pearson correlation that were non-calculable, significant and negative, significant and positive, non-significant and positive and non-significant and negative in each treatment group. Pearsons correlation at 5% significance. LT is low temperature, HT is high temperature, LL is continuous light, LND light dark cycles, CF continuous feeding, DF feeding in the perceived day.



Appendix B

B. 21 Genomic annotation of CpG site in both significant and non-significant (Pearson) correlation with gene expression, at $P < 0.05$. Results are split by the direction of correlation (positive vs negative) and experimental treatment. LT is low temperature, HT high temperature, LL is continuous slight, LND is light dark cycles, CF continuous feeding, and DF is feeding in the perceived day.



Appendix C.

C. 1 Specifics on the dates of transfer and how they were used to calculate days in SW and days between FW sampling and SW transfer used for calculation of growth traits and statistical models. * indicates that the average FW sampling date was used as FW sampling was split over two days.

	Sample #	FW Sampling	SW transfer	SW sampling	Days in SW	Extra FW days*
<i>RAS</i>	1-13, 15-310					
	312-750, 774-900	1-2/11/2021	17/11/21	1/3/22	105	16.5
	2901-3001	1-2/11/2021	17/11/21	8/3/22	112	15.5
	14, 751-773	1-2/11/2021	17/11/21	7/3/22	111	15.5
<i>Loch</i>	901-1750	3-4/11/2021	9/11/21	2/3/22	114	6.5
	1751-1900	3-4/11/2021	9/11/21	3/3/22	115	6.5

C. 2 The number of SNPs removed at each stage of QC, with the start and end number of SNPs for the parental and combined FWSW offspring populations

	Step	Parents	FW-SW offspring
	Start	55,357	65,774
SNPs removed	<i>Dup/chr30</i>	878	800
	<i>HWE</i>	125	9,123
	<i><10% individual</i>	0	0
	<i><10% SNPs</i>	104	936
	<i>MAF</i>	1,093	1,127
	End	53,157	53,788

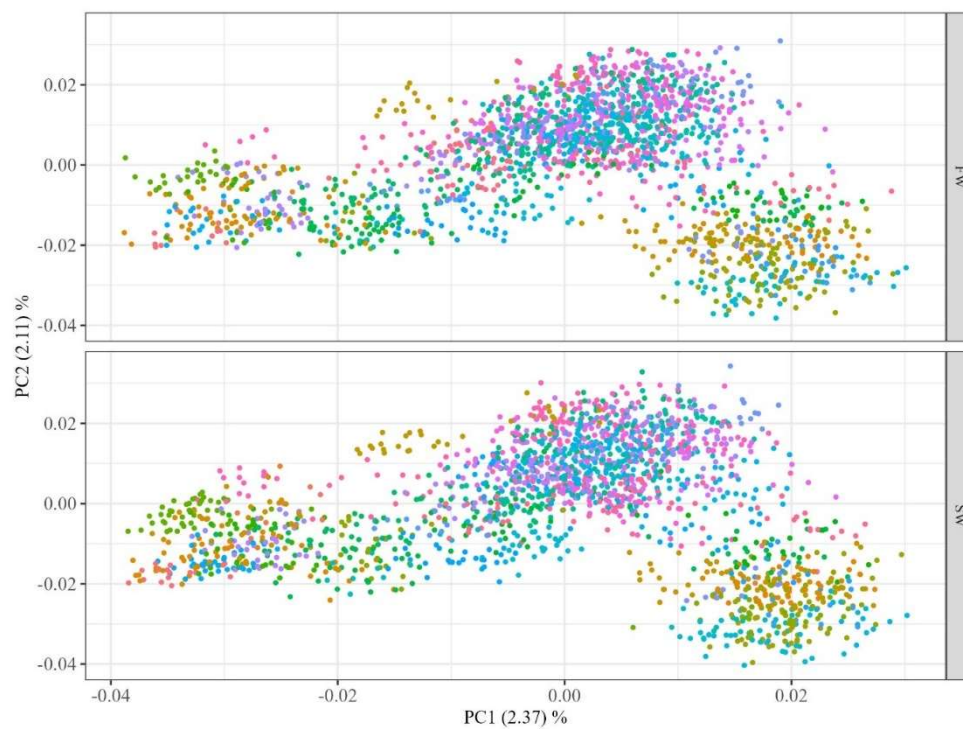
C. 3 Number of SNPs (count) and position of the final set of common filtered SNPs per chromosome

Chr	Count	Coverage (kb)
1	3,109	15,8976
2	1,018	71,370
3	1,900	92,338
4	1,708	82,398
5	1,670	80,317
6	1,678	87,014
7	1,189	57,626
8	362	26,091
9	2,299	141,543
10	2,258	115,973
11	1,595	93,888
12	1,610	91,863
13	1,970	107,731
14	1,842	93,886
15	1,795	103,896
16	1,355	87,455
17	9,16	57,656
18	1,037	70,695
19	1,378	82,833

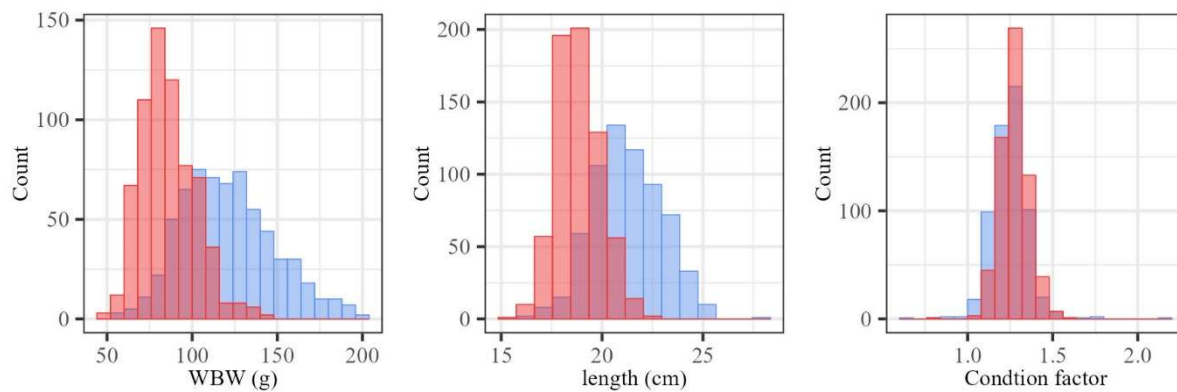
Appendix C

20	1,620	86,732
21	1,064	57,906
22	1,196	62,985
23	1,257	49,601
24	1,057	48,558
25	922	51,339
26	754	47,201
27	1,019	43,591
28	841	39,329
29	708	42,455

C. 4 Principal component analysis of genomic relationship ship matrix for all genotyped individuals, split by sampling point (salt- and fresh-water). Colour represents family ID

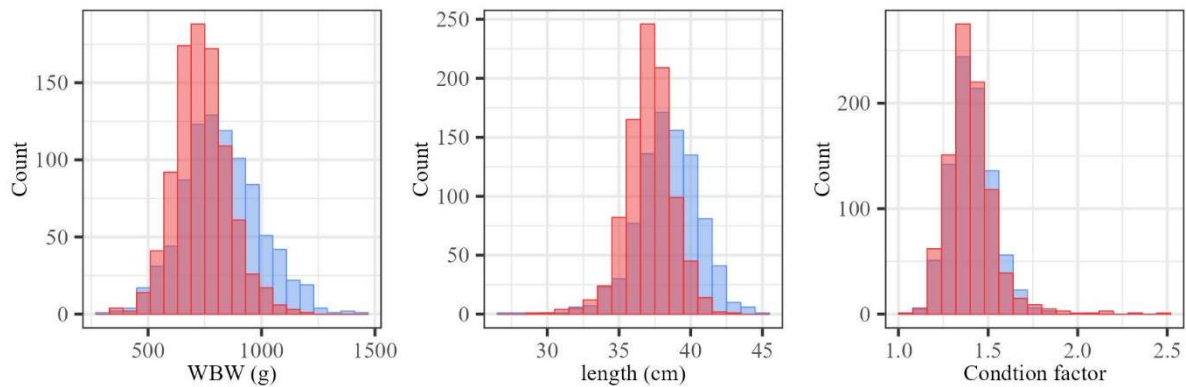


C. 5 Histogram of phenotypic traits, WBW (g), length (cm) and condition factor (k) measured at the end of the FW phase. Red indicates RAS-reared and blue loch-reared populations

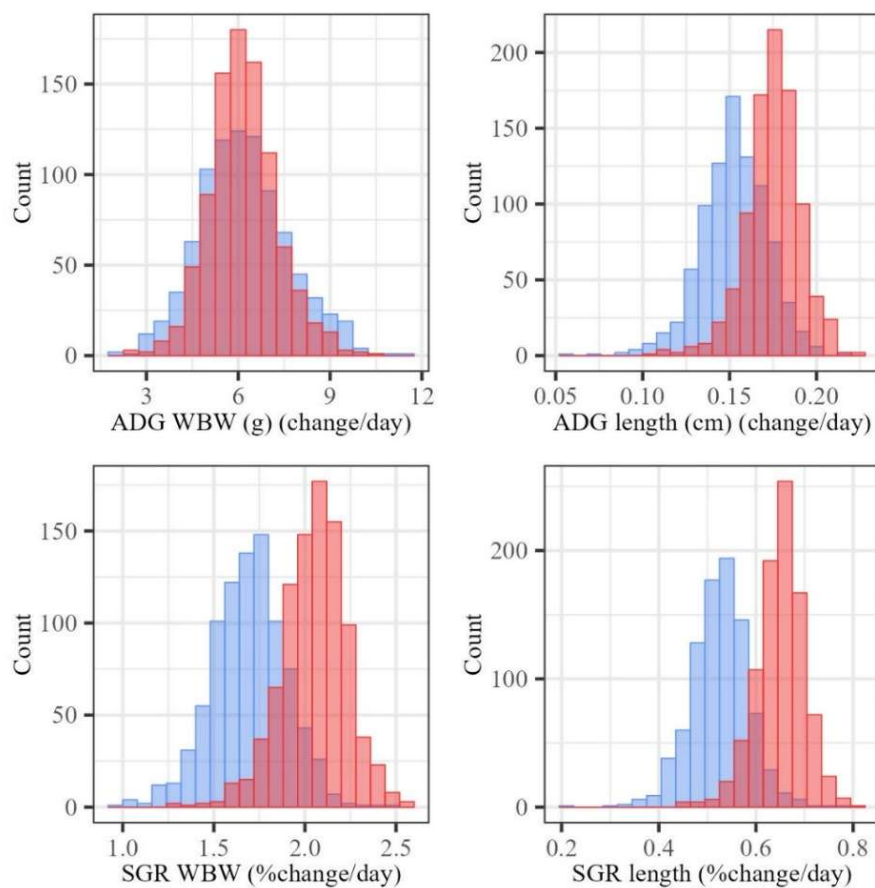


Appendix C

C. 6 Histogram of phenotypic traits, WBW (g), length (cm) and condition factor (k) measured at the end of the SW phase. Red indicates RAS-reared and blue loch-reared populations.

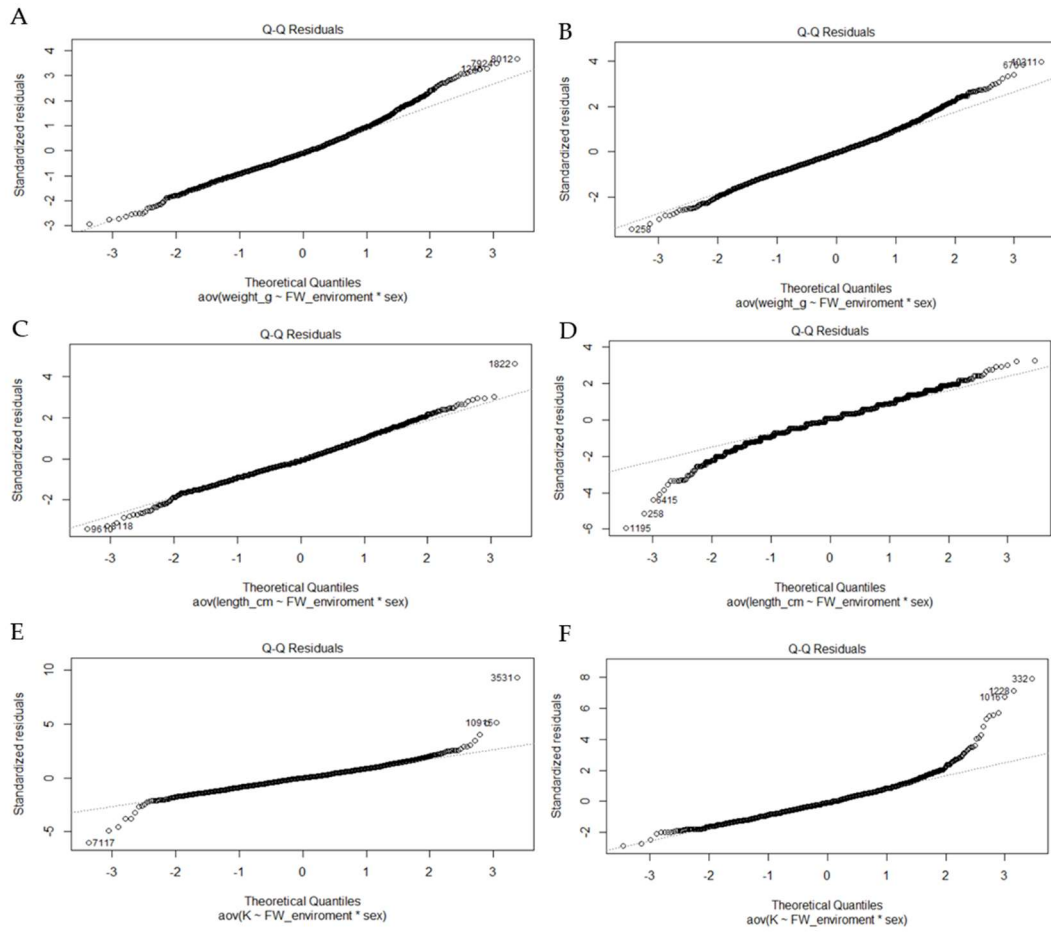


C. 7 Histogram of growth traits, average daily gain for whole-body weight (ADG WBW), average daily gain for length (ADG length), specific growth rate for whole-body weight (SGR WBW) and specific growth rate for length (SGR length), measured between the end of FW phase and up to approximately 3-months post-SW transfer. Red indicates RAS-reared and blue loch-reared populations.

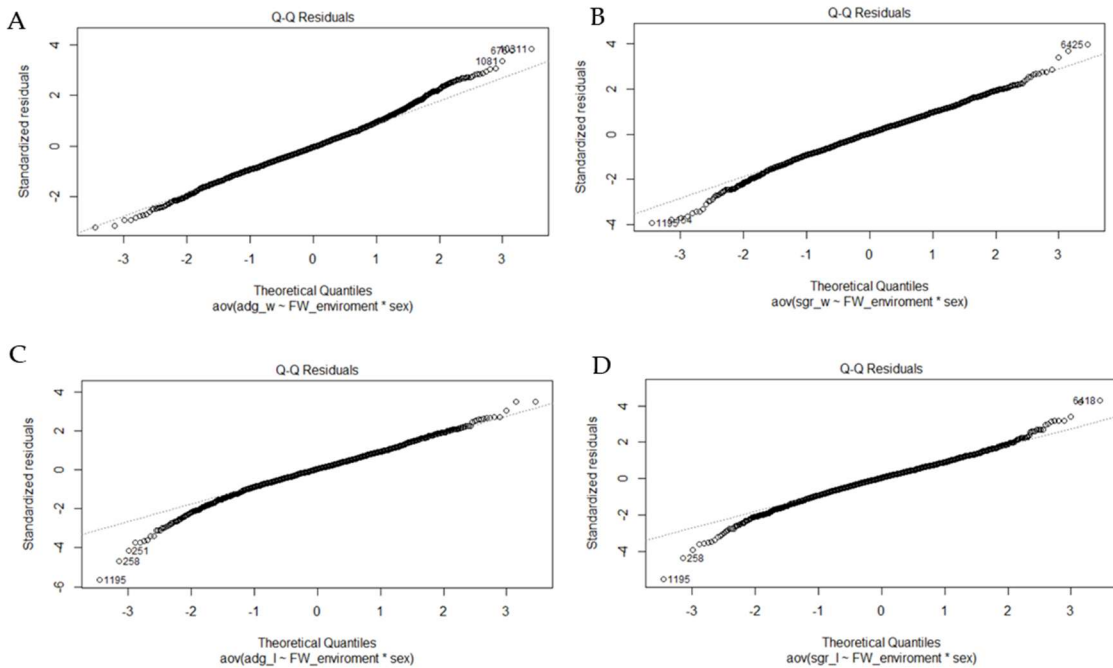


Appendix C

C. 8 Q-q plot of residuals for WBW_{FW} (A), $length_{FW}$ (C) and K_{FW} (D) as well as WBW_{SW} (B), $length_{SW}$ (D) and K_{SW} (F).



C. 9 q-q plots for ADG_{WBW} (A), ADG_{Length} (B), SGR_{WBW} (C), and SGR_{Length} (D).



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